Nanocarrier system for overcoming multidrug resistance in cancer

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“Cancer is an expansionist disease; it invades through tissues, sets up colonies in hostile landscapes, seeking “sanctuary” in one organ and then immigrating to another. It lives desperately, inventively, fiercely, territorially, cannily and defensively- at times, as if teaching us how to survive. To confront cancer is to encounter a parallel species, one perhaps more adapted to survival than we are.”

Siddhartha Mukherjee
The Emperor of all Maladies

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Manu Smriti Singh

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Dedicated to my dadaji

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1. Aim and Scope

Of all the cases, majority of cancer patients respond to therapeutic interventions transiently or incompletely. These multidrug resistant (MDR) cancer cases are either resistant to chemotherapy or acquire resistance during treatment. Overcoming MDR in cancer is a major challenge to clinicians and researchers alike. Transporter proteins of the ATP-binding cassette (ABC) superfamily are over-expressed on membranes of MDR cancer cells and are responsible for effluxing anti-cancer drugs. Amongst the ABC transporters identified from prokaryotes to humans- P-glycoprotein (P-gp), multidrug resistance-associated protein-1 (MRP1) and breast cancer resistance protein (BCRP) are the most characterized phenotypes that confer drug resistance.

An interesting phenomenon has been the emergence of routinely used pharmaceutical excipients (surface active agents) such as surfactants, poloxamers, PEG derivatives and their analogues as MDR modulators. The first chapter highlights inclusion of these approved 'Generally Recognized As Safe' pharmaceutical excipients in different class of nanocarriers.

Nanoparticles offer promise as drug delivery systems mainly due to the fact that they prevent recognition of drugs by efflux pumps present on cell membrane and improve drug accumulation at the tumor site; thereby, reducing systemic side effects or pharmacokinetic profiles of therapeutic molecules leading to killing of drug resistant tumor cells. The current arsenal of nano-sized formulations comprise of liposomes, solid-lipid nanoparticles or polymeric micelles etc., which have been developed to carry anti-cancer drugs and/ or inhibitor or a combination thereof with the sole aim of reversing MDR in cancer.

In first part of this work, we formulated nanoparticles using different excipients. The aim was to develop nanoparticles with inherent 'surface active' properties (devoid of MDR inhibitor), which following their interaction with cell membrane would modulate P-gp function so as to facilitate substrate entry. The effect of excipient-based nanoparticles was evaluated on highly drug resistant P-gp over-expressing brain tumor cell lines.

Different generations of ABC inhibitors have proved disappointing due to ineffective inhibition of efflux transporters, inherent toxicity and low availability at tumor site. It is increasingly important to prevent pharmacokinetic and pharmacodynamic interactions between anti-cancer drug and MDR inhibitors and the resultant systemic toxicity of latter. Encapsulating MDR inhibitor in nanoparticles not only improves their intra-tumoral accumulation profile, it also prolongs inhibitor release which can lead to sustained and efficient sensitization of drug resistant cells towards anti-cancer drug. In the second set of this work, inhibitor-loaded nanoparticles were investigated.

In this part, we further wanted to evaluate the effect of surface charge on the properties of MDR inhibitor-loaded nanoparticles in drug resistant and drug sensitive cell lines. Hence, first and third-generation P-gp inhibitors- verapamil hydrochloride and elacridar were encapsulated in non-ionic, cationic and anionic nanoparticles and assessed for their ability to reverse cancer drug resistance. Mechanism of endocytosis of nanoparticles in both cell lines were also investigated by using different pharmacological inhibitors.

Two recently synthesized quinazoline compounds- KCJ-160 and KCJ-199, both exhibiting inhibition of BCRP and to a lesser extent of P-gp have been identified. In the last part of my work, both broad spectrum MDR inhibitors were formulated in nanocarriers and assessed in comparison to their soluble counterparts for their potency to overcome P-gp and BCRP mediated drug resistance in relevant cell line models.

2. Theoretical Background

2.1. Multidrug resistance

A principal challenge in the fight against cancer is multidrug resistance (MDR). Analogous to the microbial drug resistance, the ability of cancer cells to resist treatment towards a vast spectrum of structurally unrelated drugs is referred to as multidrug resistance in cancer. The reason why same chemotherapeutic treatment yields different responses in patients can be attributed to the degree of resistance developed by the host's tumor cells. MDR phenotype could develop either due to exposure to chemotherapy for prolonged time (extrinsic) or genetic alterations in tumor cells (intrinsic) hampering chemotherapeutic success[1]. The energy driven pumping out of cytotoxic drugs by resistant cells leads to diminished intracellular drug concentration. It is worth noting that such resistance renders chemotherapy ineffective even when adequate dose of the drugs are administered.

Transporters are cell membrane proteins which facilitate/ regulate permeation of low molecular weight compounds and ions, driven by ATP hydrolysis. Around 48 structurally related transporters, collectively known as the ATP-binding cassette or ABC superfamily are responsible for the activity of efflux pumps and subsequent resistance against the cytotoxic drug[2]. In fact, over 50% of all cancers express detectable levels of ABC transporters[1]. These efflux proteins are expressed on cell membrane and play key physiological role in modulating absorption, excretion, metabolism and detoxification of various substrates at pharmacological barriers such as blood-brain barrier (BBB), lung, liver, kidney and gut[3]. ABC transporters have been categorized into seven subfamilies (designated as ABCA-ABCG). Three families concerned with drug transport and their associated efflux protein are 1) B-subfamily- P-glycoprotein (P-gp/ ABCB1/ MDR1), 2) C-subfamily- multidrug resistance-associated protein (MRP1/ ABCC1) and 3) G-subfamily- breast cancer resistance protein (BCRP/ ABCG2/ MXR). P-glycoprotein, a 170kDa plasma membrane glycoprotein, termed P-gp for its purported function in mediating a change in membrane permeability, is the most extensively characterized MDR transporter of the ABC family[4]. Due to its broad substrate specificity and ubiquitous expression in most excretory and

barrier functional tissue, P-gp poses serious obstacle in the treatment of infectious disease, brain disorders and cancer[5].

2.2. MDR Inhibitors

Soon after MDR was discovered, intensive screening of drugs already approved for other medical purposes that could have putative MDR modulating effects began. Many first, second, and third generation potent MDR modulators/ inhibitors have undergone laboratory testing since then[6] (Fig. 1). This has been followed by clinical trials in various combinations with anti-cancer drug to enhance the cytotoxicity against tumor cells.

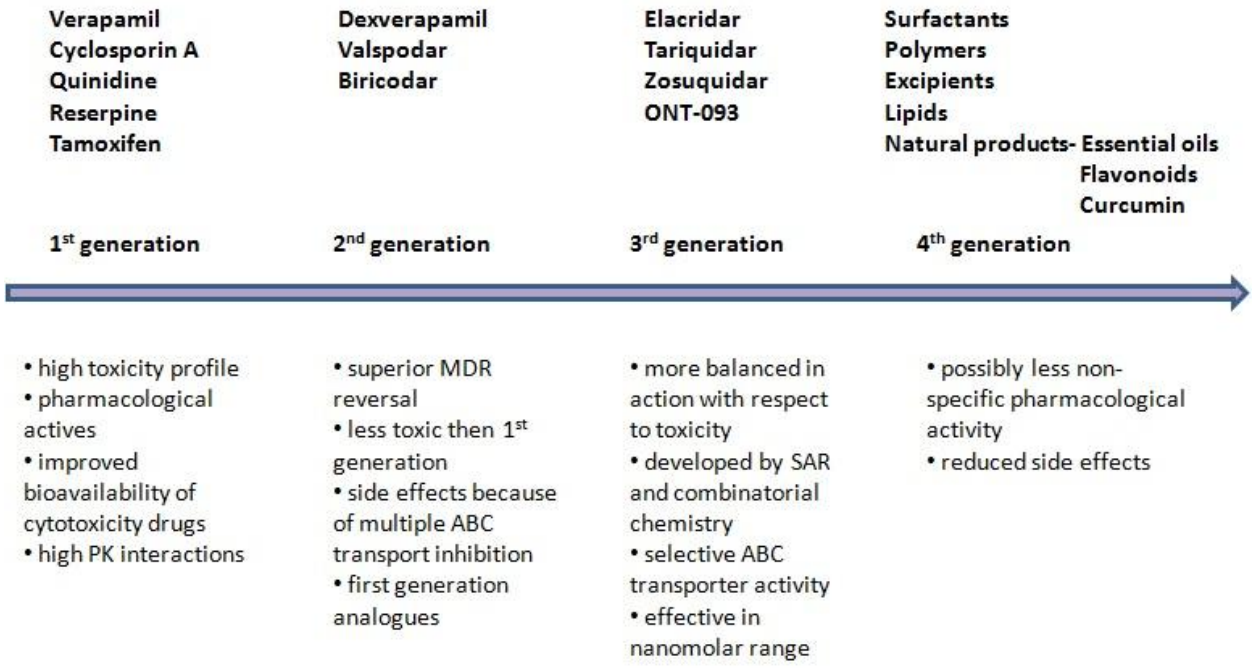

Figure 1: Timeline of development of MDR inhibitors

Cyclosporine A, for example an immunosuppressant and verapamil, a calcium channel blocker was evaluated as the 'first generation' MDR inhibitors. These inhibitors exhibited high toxicity profiles at the concentration they were required to attenuate P-gp function which proved to be a limiting factor in their clinical development. First generation inhibitors showed lack of efficacy, severe toxicity and side effects. In many clinical trials that followed, neurological and

hematological toxicity were common[6]. Another drawback of these inhibitors was the chemotherapy-related toxicity.

This led to the development of 'second generation' of P-gp inhibitors which were majorly structural analogues of first generation inhibitors. Cyclosporine analog named valspodar (PSC 833) and R-enantiomer of verapamil amongst other second generation inhibitors exhibited superior MDR reversal efficacy and lower toxicity. However, the unpredictable pharmacokinetic interactions limited drug clearance and metabolism of chemotherapy leading to toxic plasma concentrations[7]. Their interactions with cytochrome P450 enzymes (which in turn metabolize certain anti-cancer drugs such as paclitaxel) lead to alteration of pharmacokinetic properties of such drugs. As an example, in a phase I trial, variation in paclitaxel pharmacokinetics was observed, when paclitaxel was administered prior to P-gp inhibitor- valspodar (PSC 833)[8]. Several inhibitors that gave promising results *in vitro*, ultimately failed in clinical trials.

'Third generation' P-gp inhibitors were designed favoring low pharmacokinetic interactions, selectivity for a specific ABC transporter and low vulnerability for metabolic transformation by cytochrome P450 3A[7]. These inhibitors such as elacridar (GF- 120918), tariquidar (XR 9576), OC144-093 (ONT-093) etc., demonstrated MDR reversal at nanomolar range. Although, many compounds are under clinical trials, most studies have been discontinued before completion due to non-achievement of improved response rate[9] or a positive benefit-risk ratio[10]. Zosuquidar (LY335979), a third-generation inhibitor when co-administered with either doxorubicin or etoposide in mice bearing P388/ADR murine leukemia cells resulted in increased life span and no apparent alteration of pharmacokinetics[11]. The inhibitor was also shown to enhance anti-tumor efficacy of paclitaxel in human non-small cell lung carcinoma xenografts over-expressing MDR transporters. Phase I trial of zosuquidar together with daunorubicin and cytarabine resulted in a ~75 % response rate of 16 patients with acute myeloid leukemia (AML) demonstrating that the inhibitor can be given safely in combination with induction doses of conventional cytotoxic drugs[12]. Subsequently, in a phase I/II study in patients with non-Hodgkin's lymphoma, minimal effects of zosuquidar were observed on the pharmacokinetics of

doxorubicin and vincristine[13]. However recent randomized phase III clinical trials in AML patients showed no overall survival although there was not much increase in toxicity [14].

Owing to failure of MDR inhibitors in clinical trials, alternative ways and techniques of overcoming MDR are being explored[15,16]. One way is to alternate and optimize chemotherapy regimen or to develop novel non-P-gp substrate anti-cancer drug. As an adjunct to chemotherapy; inhibition of MDR using peptides, monoclonal antibodies or MDR gene inactivation using small interfering RNA related studies are underway to overcome MDR at molecular level. 'Fourth generation' inhibitors are being sought after and compounds with higher potency, safe ADMET profiles and high specificity are desirable. Certain surfactants, polymers and natural compounds which are inherently 'inert' and approved for pharmaceutical applications hold the potential to form fourth generation of MDR inhibitors/ modulators.

Though the ideal compound is difficult to find in a practical sense, alternative approaches should also be explored. Here, we briefly discuss the surfactants and polymers which have consistently exhibited MDR overcoming properties. Some of the promising results are offered from the area of nanotechnology. Nanocarriers functionalized with 'inert' fourth generation excipients have shown enhanced ability to evade expulsion by ABC pumps, which is the prime focus of this review and would be covered in subsequent sections.

2.3. Pharmaceutical excipients with P-gp inhibitory activity

Recent literature shows P-gp modulating ability of several excipients routinely used in pharmaceutical formulations. They are generally added to increase solubility of therapeutic drugs and are known to display safe pharmacokinetic profiles. Different classes of these excipients such as surfactants, lipids and pluronics are reported to chemosensitize MDR-related transmembrane proteins.

Surfactants have found use as emulsifiers in NP preparation owing to their interfacial activities. They have a hydrophilic polar head and water soluble, lipophilic hydrocarbon tail. One of the initial findings on reversal of daunorubicin resistance by screening surfactants was undertaken by Woodcock et al in the early 1990's[17]. They observed that all the active MDR reversing

detergents contained (Polyethylene glycol) PEG moieties in their hydrophilic portion but bore no similarities in their hydrophobic segments such as Cremophor® EL (PEG 35), Solutol® HS 15 (PEG 660 12 hydroxystearate), Tween® 80 (PEG 20)(Table 1). Rege et al[18], reported, P-gp modulating properties of above mentioned non-ionic surfactants-Cremophor® EL, Solutol® HS 15, Tween® 80. At a concentration range of 0 to 1 mM of these surfactants, an increase in apical-to-basolateral permeability (AP-BL) and decrease in basolateral-to-apical permeability (BL-AP) of P-gp substrate rhodamine-123 was reported. Vitamin E TPGS however showed reduced BL-AP permeability of substrate, only at a concentration of 0.025 mM. The mechanism of action of Cremophor® EL and Tween® 80 was believed to be lipid bilayer fluidization. Being hydrophobic, the non-ionic surfactants tend to be less toxic on cell membranes and can be considered safe. On the other hand, P-gp inhibitory activity of vitamin E TPGS was attributed to rigidization of the lipid bilayer.

In another study, surfactant action above and below critical micellar concentration (CMC) was evaluated. With respect to P-gp inhibition, it was demonstrated that higher inhibition would be obtained if the surfactants are more active above CMC[18]. As in this case, they would not just enhance drug absorption by improving drug solubility, but as well inhibit efflux by sensitizing P-gp embedded within cellular membranes. A surfactant based *in vitro* study showed that compounds which were neither too hydrophilic nor too hydrophobic, but had an optimal hydrophilic-lipophilic balance (HLB) in the range of 10-17 resulted in uptake of the anti-cancer drug epirubicin[19]. Pre-treatment with Tween® 20, Tween® 80, Myrj® 52 and Brij® 30 significantly raised the intracellular accumulation of epirubicin in human colon adenocarcinoma (Caco-2) cell line and excised rat intestinal mucosa.

With the characterization of these excipients for their P-gp modulatory activities, there has been a considerable focus in this area of research with many new surfactants qualifying as potential P-gp modulators in the last decade. Gelucire® 44/14 and Peceol® demonstrated downregulation in P-gp expression in Caco-2 cells by decreasing rhodamine 123 drug efflux by P-gp protein thereby altering intestinal absorption[20]. Surfactants that could modify pharmacokinetics of orally administered drugs, which are also P-gp substrates were

investigated[21]. For digoxin transport in the everted gut sac technique, the most effective excipients were in the order: Labrasol > Imwitor 742 > Acconon E = Softigen 767 > Cremphor® EL>migloyl > Soluto®l HS 15 > sucrose monolaureate > Tween® 20 > TPGS > Tween® 80. They also evaluated absorption of another drug celiprolol and concluded that mechanism of transport inhibition for each drug differs.

2.4. Natural and synthetic polymers with P-gp inhibitory activity

Polymers such as polysaccharides, PEG derivatives, dendrimers, thiolated polymers and amphiphilic block copolymers were researched and reported for their P-gp modulatory activities[22,23]. Amongst natural polymers, anionic polysaccharides such as xanthan and gellan gum, dextran, fucoidan and sodium alginate have been patented and qualified as oral bioavailability enhancers and their MDR overcoming abilities[24]. The synthetic polymers are routinely used in the nanocarrier drug delivery formulations lending: 1) improved drug solubility; 2) steady state therapeutic level; 3) reduced systemic effects of incorporated drugs *in vivo.* Hence, P-gp inhibiting properties of such polymers make their use in anti-MDR, anti-cancer formulations desirable.

PEG derivatives and analogues offer wide array of applications[25] and constitute large number of potential MDR modulators[23]. Experiments were carried out to assess the P-gp inhibitory activity of PEG with different molecular weights[26]. It was reported that secretory transport of rhodamine 123 across isolated rat intestine was inhibited on adding different concentrations (0.1-20% w/v) of PEG and that it was not dependent on their molecular weights. Treatment with PEG led to an enhanced accumulation of paclitaxel and doxorubicin in Caco-2 cells[27] and higher rates of intestinal transport and absorption of prednisolone and quinidine in rats[28]. When compared to the activity of Cremophor® EL (0.1% w/v) and Tween® 80 (0.05% w/v), PEG 300 (20% w/v) was able to inhibit MDR1- Madin-Darby canine kidney (MDCK) cancer cells completely.

Nanoparticles and liposomes are generally PEGylated to impart stealth properties to them[29]. Few PEGylated liposomes have been evaluated for their ability to overcome MDR too[30,31]. In one study[19], pre-treatment of Caco-2 cells with surfactants composed of PEG and fatty acids/

fatty alcohols led to enhanced intracellular accumulation of epirubicin. In liposome-based studies which would be mentioned in subsequent sections of this review[32,33,34], PEGylation seems to improve drug uptake in drug resistant cancer cells[23]. Increased tumor exposure by long circulating liposomes mimics continuous infusion[31], imparts stealthiness and indeed PEG-mediated MDR modulating abilities of nanocarriers would add another feather to their cap[26]. It would be interesting to mention here that many of the surfactants based MDR modulating excipients mentioned in the previous section have PEG as hydrophobic head group. Some of the PEG derivatives and analogues are enlisted in Table 1.

Myrj® (polyoxyethylene 40 stearate)[19]
Brij® (polyoxyethylene lauryl ether)[35]
Peceol® (glycerylmonooleate) [20, 36]
Gelucire® 44/14 (lauroyl macrogol-32 glycerides) [20]
Labrasol® (caprylocaproyl macrogol-8 glycerides)[37]
Cremophor® EL (glycerol polyethylene glycol ricinoleate)[17]
Solutol® HS 15 (polyethylene glycol-15-hydroxystearate)[38]
Polysorbates (Polyoxyethylenesorbitanfattyacidesters)[21]
TPGS (D-alpha-tocopheryl-poly(ethylene glycol) 1000 succinate)[39]

Table 1: PEG derivatives and analogues

Most non-charged surfactants (Cremophor® EL, Solutol® HS 15, polysorbates and TPGS) exhibit a membrane anchoring hydrophobic tail and a hydrophilic headgroup. Seelig et al,[40] reviewed that these head groups act as hydrogen bond acceptors as they are rich in ester and ether groups responsible for hydrogen bonding interactions between P-gp and surfactant. Pluronics® (also known as poloxamers) are another set of polymeric pharmaceutical excipients which have been used as micellar nanocarriers for drug delivery[41,42-43].

2.5. Drug delivery systems

Use of drug delivery systems (DDS) has shown considerable progress in past few decades especially with respect to cancer treatment. Nanoparticles (NPs) are proven to accumulate in tumor mass due to hyper-permeable vasculature and malfunctioning tumor lymphatic system without affecting healthy tissues; a phenomenon referred to as the 'enhanced permeability and retention' (EPR) effect [44]. Nanocarriers in turn, offer promising drug delivery vehicles as they prevent recognition of drugs by the efflux transporters, readily endocytosed by drug resistant cells thereby improving intracellular drug accumulation. Different types of nanocarriers are established now such as nanoparticles, micelles, liposomes, nanogels and other self-assembled nanostructures (Fig. 2). These drug delivery systems can be fine-tuned by surface functionalization with ligands for active targeting[45]. Nanocarrier-based strategies to overcome cancer drug resistance have shown promising outcomes in the last decade[46,47].

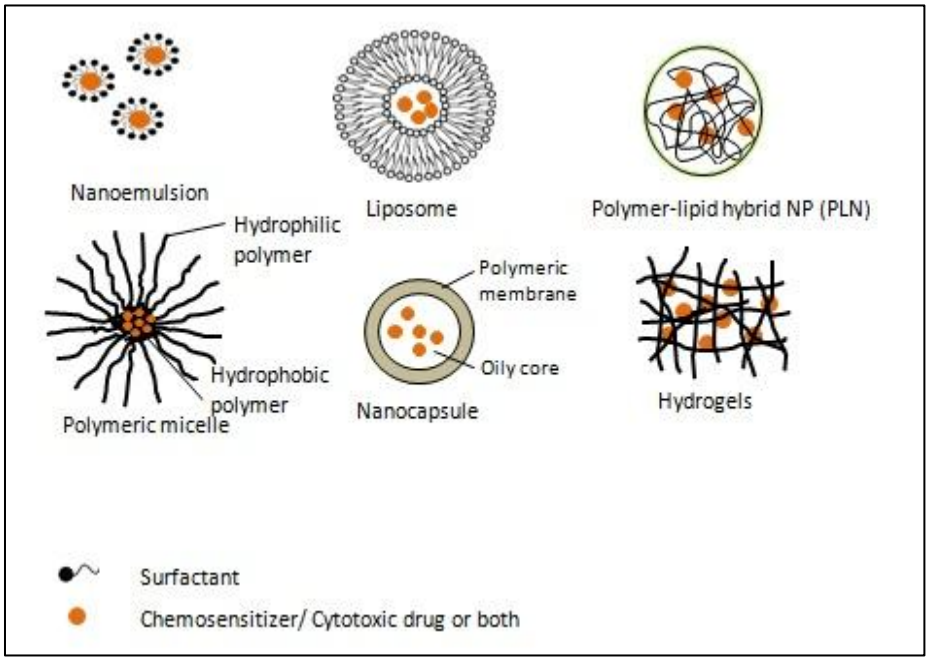

Figure 2: Drug delivery systems such as nanoemulsion; liposome; polymer lipid hybrid NP; polymeric micelle; nanocapsule; hydrogels used to address multidrug resistance in cancer

Most chemotherapeutic studies, on co-delivery of P-gp substrate together with P-gp inhibitors have failed at several stages in clinical trials. Several factors are responsible for these

therapeutic failures[6]. Due to expression of P-gp transporters at physiological barriers, inhibition of unrelated non-tumor cell P-gp transporters can compromise routine functions of body. For example, inhibition of P-gp transporters expressed at the blood-brain barrier (BBB) can cause neurological toxicity[48]. In such cases, decreased systemic clearance of anti-cancer drug has led to higher frequency of adverse effects associated with chemotherapy. Pharmacokinetic and pharmacodynamic interaction between MDR inhibitors and anti-cancer drugs are the most difficult to assess. In this scenario, the ability of nanoparticles to localize at tumor tissue (due to EPR effect) can reduce the level of exposure of inhibitors to non-target ABC transporters expressed at other physiological barriers. Inhibitors in soluble form are ineffective due to their low availability at tumor site which can be additionally addressed by delivering inhibitors via nanocarriers. Their sustained release at the tumor site can provide sufficient inhibition of ABC transporters to overcome drug resistance.

On the other hand, inhibition of a specific transporter can be accompanied by inhibition of a non-target transporter. Tariquidar, a third generation inhibitor at a concentration of >100nM was shown to cross react with breast cancer resistance protein (BCRP) along with P-gp inhibition[49]. This has severe implications on healthy tissues such as intestine or BBB where BCRP expression plays protective roles. Action of biotransformation enzymes such as cytochrome P450 can also be compromised as certain P-gp inhibitors are substrate of cytochrome P450 as well [50]. Delivery of inhibitors as nano-formulation has the potential to reduce such non-specific interactions causing unrelated pharmacological activities but more *in vivo* studies are required in this direction.

Therapeutic DDS are promising alternative to conventional chemotherapeutic drug/ inhibitors. A number of features such as narrow size distribution, increased solubility and stability of drug molecule, high drug loading efficiency, controlled release, enhanced blood circulation time and feasibility to deliver multiple such therapeutic agents in single formulation have aided in targeting tumor cells, selectively delivering drug payload. Co-administration of anti-MDR and an anti-cancer drug in nanocarriers has demonstrated significantly enhanced cytotoxicity[42]. Several studies have established better pharmacokinetic profiles of the anti-MDR drugs when

administered in *in vivo* tumor models as compared to the animal group where the drug was administered in soluble form[41,51].

2.6. Categorization of nanocarriers on the basis of functionality

Different combination therapies have been explored in order to overcome MDR (Fig. 3). On the basis of research work in last few decades a pattern has emerged. The conventional chemotherapy co-administered with one of the three generations of MDR inhibitors (Fig.3a) for one, are yet to yield positive clinical outcomes in terms of overall survival and progression free survival. Scientists have been relentlessly working to develop drug delivery systems and combination treatments with sole aim to neutralize MDR-mediated drug efflux, built-up intracellular anti-cancer drug concentration and subsequently kill the tumor cell with minimum side effects in terms of toxicity and pharmacokinetic interactions on the body. A detailed case by case analysis of nano-based combination therapies is discussed to have a better overview of the most promising strategy that could be chalked out to overcome MDR in future. Inherent MDR modulating/ inhibiting nanocarriers form fourth category (Fig. 3e).

i) P-gp substrate/ anti-cancer drug loaded nanocarrier co-administered with free MDR-inhibitor (Fig.3b)
ii) MDR-inhibitor encapsulated nanocarrier co-administered with free anti-cancer drug (Fig.3c)
iii) Anti-cancer drug and MDR-inhibitor both co-encapsulated in the nanocarrier (Fig.3d)
iv) Anti-cancer drug and/or P-gp inhibitor encapsulated in MDR-reversing nanocarriers (Fig. 3e)

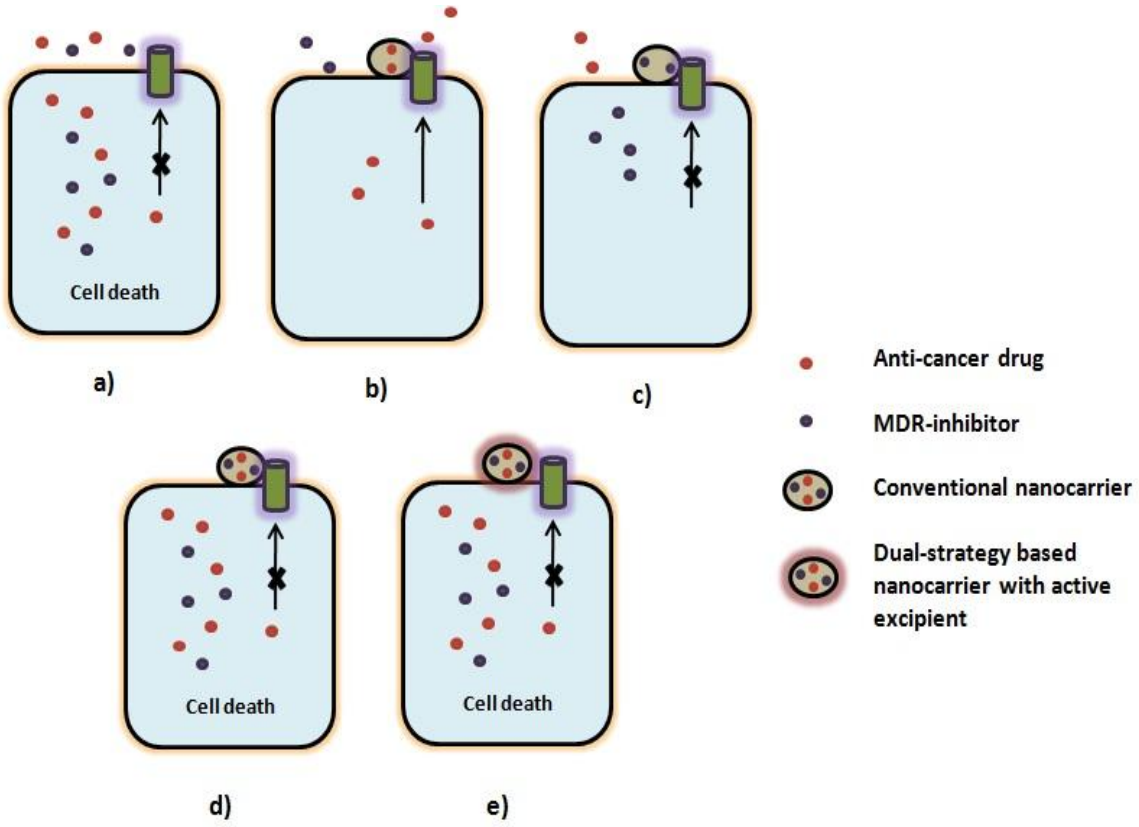

Figure 3: Combination strategies of MDR inhibitor, anti-cancer drug and nanocarriers to circumvent multidrug resistance in cancer

2.6.1. P-gp substrate/ anti-cancer drug loaded nanocarrier co-administered with free MDR-inhibitor (Fig.3b)

In a pharmacokinetic study on male SD rats, self-microemulsifying drug delivery systems (SMEDDS)[51] exhibited significant area under the curve above therapeutic level of paclitaxel as compared to the free paclitaxel wherein both groups were co-administered with free cyclosporin A. This enhanced systemic exposure of orally administered paclitaxel together with cyclosporin A was due to a diminished clearance and an increased uptake which enhanced oral bioavailability of paclitaxel.

Likewise, Krishna et al, reported that doxorubicin-loaded liposomes can reduce anti-cancer drug-valspodar interactions leading to tumor growth inhibition in BDF1 mice with resistant lymphocytic leukemia (P388/ADR)[52]. Increased delivery of liposomal doxorubicin to tumor site,

alleviation of pharmacokinetic alterations and subsequent protection from toxicity exacerbation were associated with anti-tumor activity. When additional stealth properties were imparted to doxorubicin liposomes following PEGylation, an even higher tumor suppression were reported by the same group[53]. In human breast cancer tumor xenograft model (MDA435LCC6), sterically stabilized liposomes showed a further decrease in doxorubicin area under the curve circumventing pharmacokinetic interaction with soluble valspodar.

This strategy, however, does not resolve the unfavorable pharmacological properties exhibited by MDR inhibitors administered in soluble form. Low availability of inhibitors at tumor tissue and ineffective inhibition of a specific transporter will ensue. This combination strategy does not rule out the possibility of unrelated pharmacological activity and will eventually lead to non-target transporter inhibition.

2.6.2. MDR-inhibitor encapsulated nanocarrier co-administered with free anti-cancer drug (Fig.3c)

In order to avoid the pharmacokinetic interaction between anticancer drug and valspodar (PSC 833), Lo et al,[54,55] evaluated its inhibitory activity as free, liposomal and intralipid (o/w emulsion) formulations on the uptake and transport of epirubicin in Caco-2 cells and everted gut sacs of rats. It was observed that valspodar in free and liposomal formulations increased the apical to basolateral absorption of epirubicin in Caco-2 monolayers, thereby improving mucosal to serosal absorption of the drug in rat jejunum and ileum. However, valspodar loaded liposome achieved highest uptake of epirubicin at all studied concentrations in Caco-2 cells.

In a similar work, Binkhathlan et al, studied pharmacokinetic interactions of valspodar encapsulated (PEO-b-PCL) micelles and doxorubicin *in vivo*[56]. Intravenous administration of free valspodar along with doxorubicin led to 50% reduction in clearance (CL) of the anti-cancer drug. On the other hand, micellar formulation of valspodar reduced doxorubicin CL by only 6%, as well as a significant increase in AUC and t½ of doxorubicin were observed. Hence, this strategy of using inhibitor-loaded nanocarriers and anti-cancer drug in solution form can considerably reduce the pharmacokinetic interactions between the two drug molecules. On the other hand,

due to higher doses of administered free anti-cancer drug, the undesirable toxic effects on healthy/ non-specific tissues cannot be ruled out.

2.6.3. Anti-cancer drug and MDR-inhibitor both co-encapsulated in the nanocarrier (Fig.3d)

A number of formulations co-delivering P-gp substrates and P-gp inhibitors under various categories have shown their ability to bypass drug efflux transporters. Some promising formulations in various DDS discussed subsequently include nanoparticle[57], nanoemulsions[58], liposomes[59,60] and solid lipid nanoparticle[42]. The *in vivo* behavior of therapeutic agent/drug gets altered, following its encapsulation in nanocarriers and amongst other factors, depends on surface properties of the DDS rather than its own chemical structure. Most studies report reduced adverse effects and better therapeutic efficacy in comparison with non-targeted classical drug combination modalities used by clinicians[61].

2.6.4. Anti-cancer drug and/or P-gp inhibitor encapsulated in MDR-reversing nanocarriers (Fig. 3e)

An interesting set of nanocarriers has emerged which have ingredients/ excipients/ polymers[23] to bypass MDR drug efflux that can inherently modulate MDR transporters without co-administration or co-encapsulation of P-gp inhibitor. These nanopharmaceutical components of nanocarriers, fall under the category of 'Generally Recognized As Safe' (GRAS) substances approved by U.S. Food and Drug Administration[22].

Most routinely evaluated nanoparticles are developed using poly (lactide-co-glycolide) (PLGA) or poly(lactic acid) (PLA) as the polymer and poly(vinyl alcohol) (PVA) as the surfactant. As in most studies, NP carrying anticancer drug was not sufficient to overcome MDR until a sustained dose of P-gp inhibitors were maintained[62]. Thus, a novel strategy could involve replacing routinely used polymer/ surfactants/ stabilizers with active excipients which although inert, possess intrinsic P-gp modulating abilities[63]. In one study, replacing PVA with Pluronic® PF127 as surfactants in doxorubicin loaded PLGA NPs led to higher toxicity and overcoming of cancer MDR[64]. In a number of studies reported in this review article, P-gp substrate/ anti-cancer drug

were encapsulated in various nanocarriers and tested for their anti-MDR efficacy *in vitro* and *in vivo*. These formulations and the active MDR-reversing component used in nanocarrier preparation are enlisted in Table 2.

Formulation	MDR-reversing agent	P-gp substrate	Reference
Nanoparticle	Vitamin E TPGS	Paclitaxel	Zhang[65]
Nanoparticle	Brij® 78	Paclitaxel	Koziara[66]
Solid Lipid NP	Solutol® HS 15	Doxorubicin	Kang[67]
Lipid Nanocapsule	Solutol® HS 15	Etoposide	Lamprecht[68]
Lipid Nanocapsule	Solutol® HS 15	Paclitaxel	Roger[69]
Microgels	Pluronic® P85	Doxorubicin	Bromberg[70]
Polymeric micelles	Pluronic® P85	Doxorubicin	Sharma[41]
Amphiphilic micelles	Vitamin E TPGS	Rhodamine-123	Dabholkar[43]

Table 2. Formulations studied for overcoming MDR in cancer, with the MDR-reversing component of nanocarriers and the P-gp substrate encapsulated.

2.7. Nanocarrier system to overcome P-gp mediated drug efflux

Case wise study of various formulations is done highlighting results on *in vitro* and *in vivo* cancer drug resistant cell lines and animal models. These different classes and subclasses of nanocarriers (Fig. 3) are discussed in relation to their advantages in overcoming multidrug resistance in cancer. Their beneficial roles could be ascribed to the inherent properties to bypass and modulate the P-gp efflux system or to the surfactants/ polymers which were employed to develop them. In all these studies, the association of a P-gp modulator or a P-gp substrate with a drug delivery system reduces the effective therapeutic dose thereby improving solubility, subsequent release and bioavailability of these agents at the pharmacological sites of action.

2.7.1. Nanoparticle

Unlike aqueous core of liposomes, solid polymeric nanoparticles have been deployed to deliver hydrophobic therapeutic molecules. NPs are biodegradable, exhibit uniformity in size and shape and offer a controlled drug release profile over a period of time. Chavanpatil et al, studied effect of paclitaxel-loaded PLGA NPs and found them to be susceptible to P-gp mediated efflux on drug resistant breast cancer NCI-ADR/RES cells[62]. Resistance could be reversed with treatment of P-gp inhibitor verapamil but a sustained inhibition of P-gp was needed for therapeutic efficacy. The group later developed an AOT-alginate NPs (without inhibitor)to overcome resistance in MDR cells[57]. Aerosol OT (AOT) an anionic surfactant and sodium alginate, is a naturally occurring polysaccharide polymer used in DDS. Doxorubicin loaded AOT-alginate NP demonstrated significant and sustained enhancement of drug-induced cytotoxicity in NCI-ADR/RES cells with an increase in the level of cellular and nuclear drug accumulation. An increase in the level of cellular accumulation was observed even with a mixture of blank AOT-alginate NP and rhodamine. The results could also be translated in bovine brain microvessel endothelial cells (BBMECs) which are primary cells over-expressing P-gp. In a recent work, cyclosporine A coated-doxorubicin loaded PLGA NPs were able to improve the survival rates of A549 tumor bearing mice demonstrating the necessity to incorporate inhibitors within particles to achieve inhibition[71].

Nanoparticles of poly(lactide)–vitamin E TPGS (PLA–TPGS) copolymers were synthesized by a dialysis method[72]. D-alpha-tocopheryl-PEG 1000 succinate (TPGS) is a water-soluble derivative of natural vitamin E and is an effective emulsifier, pore-forming agent, absorption enhancer and bioavailability promoter[39]. PLA–TPGS NPs showed significant increase in the cellular uptake by 1.8- and 1.4-fold as comparison to PLGA NPs cultured with HT-29 and Caco-2 cells, respectively. The IC50 of the PLA–TPGS NP formulation with HT-29 cells was found to be 40% lower than of Taxol® at the same dose of paclitaxel. In subsequent experiments, series of particles with varying PLA: TPGS ratios were evaluated[65]. It was observed that the PLA–TPGS NPs of 89:11 PLA: TPGS ratio achieved the best effects on the cellular uptake and the cancer cell mortality of the drug-loaded PLA–TPGS NPs. *In vivo* evaluation in mice model, docetaxel-loaded vitamin E

TPGS-PLGA NP showed 284-fold efficacy (as compared to free drug) in formulations with 20% TPGS[73].

2.7.1.1. Lipid-based nanoparticles

Lipid nanoparticles encapsulating paclitaxel, with emulsifying wax as the oil phase and polyoxyethylene 20-stearyl ether (Brij® 78) as the surfactant were developed[66]. These paclitaxel nanoparticles were able to overcome P-gp–mediated resistance *in vitro* in a human colon adenocarcinoma cell line (HCT-15) and showed 9 fold lower IC50 values in paclitaxel–NP treated group than Taxol® solution. Similar results were obtained *in vivo* in a nude mouse HCT-15 xenograft model where paclitaxel NP group showed marked anti-cancer efficacy following intravenous injection.

Paclitaxel and doxorubicin loaded lipid-based NPs were also developed by another group[35]. These drug loaded NPs containing the surfactant- Brij® 78 showed 6- to 9-fold reduction in IC50 values in ovarian carcinoma NCI/ADR-RES and human melanoma cell line MDA-MB-435/LCC6MDR1. They demonstrated increased uptake and prolonged retention of drug in all lipid-based NP formulations. Maximal efficacy was observed with paclitaxel NPs prepared with Miglyol® 812 in oil phase and TPGS and Brij® 78 as surfactants (PX BTM NPs). PEGylated PX BTM NPs significantly inhibited tumor growth *in vivo* in mice bearing resistant NCI/ADR-RES cell xenografts in comparison to all tested controls.

While Koziara et al[66] reasoned out enhanced delivery and anti-angiogenic effect as the factors overcoming MDR, Dong et al[35] proved that the use of Brij® 78 for the preparation of microemulsion precursor was responsible for the same. Calceinacetoxymethylester and ATP assays confirmed that both free Brij® 78 and blank NPs inhibited P-gp and depleted ATP temporarily and reversibly. The change in the mitochondrial potential and mitochondrial swelling though transient were seen to be dominant only in MDR cells, indicating Brij® 78 and NP's influence on the mitochondrial respiratory chain.

Solid lipid nanoparticles (SLN) are lipid-based formulations with low toxicity, and have high partition for lipophilic drugs in the lipid phase. In one study, doxorubicin complexed with

soybean-oil-based anionic polymer and dispersed with lipid in water to form doxorubicin-loaded solid lipid nanoparticles (Dox-SLNs)[74]. This SLN system led to significantly higher cellular doxorubicin uptake and retention by both human (MDR435/LCC6/MDR1) and murine (EMT6/AR1.0) P-gp-over-expressing breast cancer cell lines compared to doxorubicin solution treatment. An 8-fold increase in suppression of MDR 435/LCC6/MDR1 cell colony formation was also reported. The blank SLN and the excipients exhibited little cytotoxicity. Similar positive outcomes with Dox-SLNs were reported recently in two separate studies in *in vivo* cancer models[67,75].

Polymeric lipid nanocapsule (PLN), are modified SLN where anionic polymer is incorporated into lipids to complex the drug so as to increase its partition in the lipids. In another similar study, third generation P-gp inhibitor GG918 (elacridar) was co-administered with or without doxorubicin[42]. Dual loaded PLNs showed highest cytotoxicity, doxorubicin uptake by P-gp over-expressing human breast cancer cell lines (MDR435/LCC6/MDR1) and long term suppression of cancer cell proliferation. On the other hand, administration of either component via PLN had least effect on cell death suggesting simultaneous delivery to same cellular location is critical in determining therapeutic efficacy of anticancer-chemosensitizer combination.

2.7.1.2. Poloxamer-based nanoparticles

Poloxamers also known by their trade names- pluronics are inert block co-polymers comprised of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks arranged in an A-B-A tri-block structure: PEO-PPO-PEO[76]. Their surfactant properties allow them to self-assemble into micelles with a hydrophobic core and a hydrophilic shell at concentrations above CMC. MDR reversing activities of two of these block co-polymers- Pluronic® L61 and Pluronic® P85 were demonstrated using P-gp substrates- doxorubicin and daunorubicin respectively[77,78]. SP1049C which are mixed micelles of Pluronic® L61 and F127 incorporating doxorubicin have been extensively tested *in vitro* as well as in clinical trials with positive outcomes[79]. A combination of experiments examining the kinetics, concentration dependence, and directionality of P85 effects on P-gp-mediated efflux in bovine brain microvessel endothelial cells (BBMEC) showed that both energy depletion and membrane fluidization

(inhibiting P-gp ATPase activity) were critical factors contributing to the activity of the block copolymer[80]. SP1049C has demonstrated promising results in clinical trials in patients with advanced metastatic adenocarcinoma of the esophagus and gastroesophageal junction[81] and has now been approved for chemoresistant gastric cancer[82].

Another block co-polymer, Pluronic® P85 was recently evaluated to assess its MDR preventive properties[41]. *In vitro* studies on murine lymphocytic leukemia cells (P388) cells and *in vivo* experiments on BDF1 mice bearing P388 ascite showed improved cytotoxicity when treated with doxorubicin/P85. Through various gene expressions profiling analysis, it was observed that apart from mdr1 gene, P85 abolished alterations of genes implicated in apoptosis, drug metabolism, stress response, molecular transport and tumorigenesis. Saxena et al[83], have shown poloxamer 407/TPGS mixed micelles as a delivery system for gambogic acid(GA) to overcome MDR in cancer. Cytotoxicity of GA-loaded micelles was found to be 2.9 times higher in multidrug-resistant NCI/ADR-RES cells, and 1.6 times higher in MCF-7 cells, as compared to GA administered in free form.

2.7.2. Amphiphilic micelles

Polymeric particles are generally prepared by the self-assembling amphiphilic diblock copolymer-based strategy. Amphiphilic micelles of PEG_{2000}-phosphatidyl ethanolamine (PEG-PE)/ vitamin E TPGS solubilized with a P-gp substrate rhodamine-123 were developed making use of the P-gp altering properties of non-ionic surfactant TPGS[43]. The internalization of these micelles by Caco-2 cells, opposite to the internalization of the free rhodamine-123, was not influenced by the P-gp inhibitor verapamil, exhibiting a P-gp-independent micelle internalization. The study hence emphasized non-requirement for co-administration of P-gp inhibitors and that the use of a P-gp inhibitor is not necessary if the DDS is sufficient to bypass P-gp related drug efflux.

Elamanchili et al[84] prepared low molecular weight amphiphilic diblock co-polymer of methoxypolyethylene glycol-block-polycaprolactone (MePEG-b-PCL) for chemosensitization of MDR cancer cells. The studies of these polyether-polyester based co-polymer showed higher accumulation of P-gp substrates- rhodamine-123, doxorubicin and paclitaxel in Pgp over-

expressing human ovarian cancer cell line NCI/ADR-RES, P-gp transfected canine kidney cell line MDCKII-MDR1 and parental cell line MDCKII but no influence on non P-gp expressing cells. Similar nanopreparation with dual functionality of paclitaxel-loaded MePEG-b-PCL micelles-delivering high doses of drugs and modulating P-gp were shown to be the reason for positive outcomes[85]. The reduction in accumulation of P-gp substrates in MDR cells at higher concentration of diblock co-polymer has been attributed to partitioning of the drug into micelles which decreases the free drug concentration available for uptake[86].

In order to improve solubility of valspodar and to aid its oral and intravenous administration in rats, methoxy-poly(ethylene oxide)-block-poly(e-caprolactone) (PEO-b-PCL) micelles were prepared[87]. These PEO-b-PCL formulations showed significant plasma area under the curve (AUC) and lower volume of distribution (Vdss) and clearance (CL). The study emphasized that the administration of a P-gp modulator in micellar form improves PK profile of the former and reduce the pharmacokinetic interactions with P-gp substrates and toxic profiles imposed by carrier such as Cremophor® EL. *In vivo* studies were subsequently done by the group in Sprague-Dawley rats[56]. Doxorubicin was administered intravenously together with cyclosporine A/ valspodar as conventional or micellar formulation. Overall, encapsulation of valspodar in polymeric micelles was shown not just reduce their effects on the clearance of doxorubicin in rat but solved solubility issues of the highly hydrophobic derivative of cyclosporine A.

2.7.3. Liposomes

Liposomes are nanovesicles which are formed spontaneously when amphiphilic lipids are dispersed in water as an internal aqueous core surrounded by hydrophobic lipid layers. Consequently, hydrophilic drugs are encapsulated within core while lipid-soluble drugs organize within the lipid membrane. With the sole aim of overcoming MDR in cancer, few liposomal formulations have been studied. In another work, transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil (Tf-L- DOX/VER) were developed and evaluated for their MDR reversal efficacy[59] in doxorubicin resistant K562 cells (K562/DOX).Tf-L- DOX/VER showed 5.2 and 2.8 times greater cytotoxicity than non-targeted liposomes (L-DOX/VER) and Tf-targeted liposomes loaded with DOX alone (Tf-L-DOX) respectively.

Another doxorubicin and verapamil co-loaded liposomes was tested for *in vitro* cytotoxicity on MDR rat prostate adenocarcinoma Mat-LyLu-B2 (MLLB2) cell lines[60]. The toxicity of relevant formulations were in the following order: doxorubicin/ verapamil co-encapsulated stealth liposomes (DARSL's) (0.0079 μM) > doxorubicin liposomes and verapamil liposomes (0.0099 μM) > doxorubicin liposomes with free verapamil (0.96 μM). The *in vivo* results[88] clearly demarcated doxorubicin clearance when administered (as liposomes) with verapamil either free or co-encapsulated, as against free administration of both molecules. The DARSL's treatment resulted in lowered doxorubicin distribution in heart, kidney, liver and lungs. Co-encapsulation of third generation P-gp inhibitor tariquidar together with paclitaxel in stealth liposomes has also shown promising *in vivo* results[33]. Treatment to human ovarian adenocarcinoma SKOV3TR cells led to enhanced cytotoxicity at a paclitaxel dose, which was ineffective in absence of tariquidar, implying significant reversal of MDR towards paclitaxel. In MDR promyelocytic leukemia-HL60 xenograft mice, gradual shrinkage of tumor was reported when treated with stealth liposomes co-encapsulating topotecan and amlodipine[34].

2.7.4. Lipid-based formulations

2.7.4.1. Nanoemulsion

Nanoemulsion refers to heterogenous mixtures of oil-in-water using high energy emulsification methods, where oil droplets are in the range of 20-200nm. Paclitaxel-nanoemulsion were developed to enhance the oral bioavailability of the anti-cancer drug: comprised of pine nut oil as internal oil phase, egg lecithin as primary emulsifier and water as the external phase[89]. They further developed nanoemulsion by co-encapsulating paclitaxel and curcumin[58]**.** Western blot results showed decrease in P-gp expression of ovarian adenocarcinoma MDR phenotype SKOV3TR cells that explained 1.8 fold reductions in IC50 values of nanoemulsions as compared to those with paclitaxel alone.

2.7.4.2. Lipid Nanocapsules

Lipid nanocapsules (LNC) refer to DDS whose structure is a hybrid between polymeric nanocapsules and liposomes[90]. They have an oily core surrounded by a tensioactive rigid membrane. Cytotoxic drug- etoposide loaded LNC showed higher efficiency than the drug

solution on glioma cells, while blank LNCs were found to be less inhibitory than the pure drug at equivalent concentrations. In a similar work, paclitaxel-loaded LNCs were shown to reduce the survival of 9L and F98 cells significantly in comparison to free Taxol® treatment. LNCs greatly reduced tumor mass in *in vivo* F98 subcutaneous glioma mice model. This study demonstrated that the inhibition of MDR efflux pumps could be due to its interaction with released free intracellular Solutol® HS-15 (an LNC component) and redistribution of intracellular cholesterol. Solutol® HS 15, a non-ionic surfactant has been proved to exhibit P-gp inhibitory activity[91]. Lipid nanocapsules have also led to improved gastrointestinal crossing of paclitaxel in Caco-2 cells via transcytosis[69].

2.7.5. Self-emulsifying drug delivery systems

Self-emusifying drug delivery systems (SEDDS) are isotropic mixtures of oils and surfactants which can then disperse in gastrointestinal lumen forming microemulsions. They can readily enhance the oral bioavailability and hence absorption of lipophilic drugs[92]. Use of such self-microemulsifying drug delivery systems (SMEDDS) comprised of vitamin E in oil phase and deoxycholic acid sodium salt, TPGS and cremophor RH 40 as surfactants has been evaluated[51]. The aim was to increase solubility of paclitaxel and evaluate efficacy of formulation when delivered paclitaxel with or without P-gp inhibitor, cyclosporine A. Compared to Taxol®, the oral bioavailability of paclitaxel SMEDDS increased by 28.6% to 52.7% at various doses. Following co-administration with cyclosporine A, paclitaxel SMEDDS showed a higher bioavailability and much longer retention time above the therapeutic level than Taxol® alone. Thus, significant improvement in paclitaxel absorption could be attributed to the combination of P-gp inhibiting lipidic excipients together with specific P-gp inhibiting drug[93].

2.7.6. Gels

Gel refers to cross-linked hydrophilic and/or hydrophobic polymer network that spans the volume of liquid medium. Their extraordinary swelling and de-swelling ability upon external stimulation such as pH, temperature etc., have found their usage in selective drug delivery applications amongst others. They can adsorb large quantities of drugs and biomolecules

(enzymes/ growth factors etc.) within their three-dimensional mesh-like structures, acting as reservoirs and releasing their cargo in a controlled fashion over a period of time.

2.7.6.1. Nanogel

NanoGel™ are synthesized by cross-linking cationic (polyethyleneimine (PEI)) with non-ionic (carbonyldiimidazole-activated (PEG)) polymer using emulsification/solvent evaporation technique. NanoGel™ immobilized with anti-sense phosphorothioate oligonucleotides (SODN)-specific to human mdr1 gene[94] demonstrated efficient transport across polarized monolayers of human intestinal epithelial cells (Caco-2) was demonstrated.

Another nanogel formulated by the same group was prepared by complexing fludarabine-PEI in the core surrounded by hydrophilic polyethylene glycol (PEG) envelope[95]. For increased internalization folate molecules were attached to the nanogels. An enhanced cytotoxicity towards MCF-7 was observed and transcellular transport of the folate-nanogel polyplexes was found to be 4 times more effective compared to the drug alone. The results showed better tumor specificity and significantly reduced systemic toxicity.

2.7.6.2. Hydrogel

Hydrogels are hydrophilic, three-dimensional networks which can imbibe large amounts of therapeutic agents[96]. Biodegradable hydrogels based on N-(2-hydroxypropyl) methacrylamide (HPMA) were able to maintain sufficient therapeutic concentrations of drug with minimal side effects. On subcutaneous implantation, release of doxorubicin, was observed up to 96 hours. In contrast to application of doxorubicin alone, a cocktail of doxorubicin with cyclosporine A blocked the proliferation of P-gp-over-expressing Bcl1 leukemia MDR cell lines *in vitro* by inducing apoptosis[97]. Promising results were obtained in mice with advanced Bcl1 leukemia as well.

2.8. Perspective and future challenges

In spite of three generations of MDR inhibitors, few positive clinical outcomes have been obtained till date. This review discusses several formulation strategies with relevant recent

examples that have resulted in: 1) increased cytotoxicity to drug resistant tumor cells in comparison to the treatment with either entity alone, 2) prolonged release of encapsulated drug/ inhibitor which leads to a sustained sensitization of resistant cells facilitating tumor cell killing by overcoming MDR, and 3) reduced systemic pharmacokinetic interactions between cytotoxic drug and MDR inhibitor.

Lipid composition of cell membrane and their interaction with drug molecules are attributed for low drug accumulation in MDR cells. Drug resistant cells differ in their biophysical characteristics in comparison to their parental counterparts. They are much rigid due to the presence of higher amount of phospholipids and saturated fatty acids in turn affecting endocytosis process; making the membranes thick and contributing to their barrier function. This in turn traps the hydrophobic anticancer drugs/ MDR substrates which get confined to the lipid bilayer before finally getting effluxed by MDR transporters. On the other hand, in sensitive cells hydrophobic drug gets transported inside the cell due to the more fluid cell membrane. Therefore, biophysical characteristics of cell membrane can aid towards a better understanding of nanocarrier-lipid bilayer interactions and more efficient mode of drug delivery to address cancer drug resistance.

Nanocarriers not only act as vehicle, they aid in accumulation of drug/ inhibitor within tumor mass which is difficult to achieve using their soluble counterparts. This tumor localization can overcome the issues of low availability at the tumor tissue and unrelated pharmacokinetic activity as observed with MDR inhibitors in several clinical outcomes. Moreover, a sustained release of cargo ensures prolonged drug delivery and sensitization of drug resistant tumors by achieving sub-optimal concentrations of therapeutic molecules. Further fine-tuning of nanocarriers is underway, to endow them with intrinsic MDR-modulating abilities.

Certain excipients/ polymers routinely used in pharmaceutical industries as well as in the preparation of nanocarriers-such as surfactants, pluronics®, PEG derivatives and analogues, amphiphilic diblock copolymers etc., are known to exhibit inherent MDR-reversing abilities. These excipients are mostly biodegradable, have safe pharmacokinetic profile, improve drug solubility and lead to an enhanced absorption as compared to the free drug. They are not

absorbed by the intestine and have been carefully studied to be safe to use in different class of formulations like liposomes, solid-lipid nanoparticles or polymeric micelles. When DDS are developed using these `surface active agents`, the surface property enables the carrier system itself to modulate ABC transporters and overcome MDR.

These compounds exhibit MDR-modulatory abilities at very low concentrations which are far-less than their clinical applications and have been approved by drug regulatory authorities for varied pharmaceutical applications; further in-depth characterization would be required to elucidate their mode of action and their interaction with plasma membranes- as nanocarriers and in free form. In some cases, free surfactant units released from nanocarriers can form micelles and entrap the co-administered hydrophobic MDR substrates which get trapped at the rigid drug resistant cell membrane reducing the efficacy of treatment. On the other hand, free surfactant units released intracellularly following nanocarrier-membrane interactions can also lead to MDR inhibition by redistributing intracellular cholesterol. Much effort should also be given to optimize the concentration to be used in surfactant-based nanocarrier formulation.

Mechanism of bypassing P-gp efflux for some of the 'surface active' excipients has been proposed to involve fluidization of lipid membrane or rigidization of lipid bilayer which could be co-related to their ability to influence nanoparticle-biophysical interactions with cell membrane lipids. Poloxamers are the most characterized in terms of understanding their MDR modulatory mechanisms. A detailed evaluation of mode of action of 'surface active agents' to overcome MDR would be desirable for better understanding of the molecular dynamics at cellular level and to avoid undesirable systemic pharmacokinetic interactions.

However, as a word of caution formulations that yield positive outcomes must be subjected to a thorough molecular assessment in order to understand the underlying mechanism of MDR inhibition. *In vitro* cell culture models must always involve a drug resistant and its parental 'sensitive' cell line to see therapeutic advantage following treatment. Benefits in terms of resistance ratio- which is the quotient of IC50 of treatment group in resistant cell line to that of untreated parental cell line, should also be reported. Characterization of cells in terms of assessing the expression profile of MDR transporter/s must be developed as a laboratory

practice. Similar studies should be conducted in relevant animal models in order to extrapolate results obtained *in vitro*.

Based on these results, novel set of nanocarriers have emerged. Formulations with 'surface active' properties loaded with both anti-cancer drug and P-gp inhibitor could be considered more viable option for overcoming MDR. The synergistic effects of P-gp inhibitor and P-gp efflux bypassing nanocarrier system can work as a 'dual strategy' which would lead to delivery of anti-cancer drug within the tumor cell, building up cytotoxic drug concentrations to the levels that can kill the target cell. Although, a detailed *in vivo* characterization of these 'inert' components needs to be done, the current set of results seems promising and can be recommended for their usage in formulating nanocarriers for their MDR reversing potentials. Simultaneously, MDR reversing surface 'active' nanocarriers should be evaluated and further explored so as to enable their clinical translatability.

2.9. References

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3. Chapter 1:

P-glycoprotein inhibition of drug resistant cell lines by nanoparticles

3.1. Abstract

Several pharmaceutical excipients are known for their ability to interact with cell membrane lipids and reverse the phenomenon of multidrug resistance (MDR) in cancer. Interestingly, many excipients act as stabilizers and are key ingredients in a variety of nano-formulations. In this study, representatives of ionic and non-ionic excipients were used as 'surface active agents' in nanoparticle (NP) formulations to utilize their MDR reversing potential. I*n-vitro* assays were performed to elucidate particle-cell interaction and accumulation of P-glycoprotein substrates-rhodamine-123 and calcein AM, in highly drug resistant glioma cell lines. Chemosensitization achieved using NPs and their equivalent dose of free excipients was assessed with the co-administered anti-cancer drug- doxorubicin. Amongst the excipients used, non-ionic surfactant-Cremophor® EL, and cationic surfactant- cetyltrimethylammonuium bromide (CTAB) demonstrated highest P-gp modulatory activity in both free solution form (upto 7-fold lower IC_{50}) and as a formulation (up to 4.7-fold lower IC_{50}) as compared to doxorubicin treatment alone. Solutol® HS15 and Tween® 80 exhibited considerable chemosensitization as free solution but not when incorporated into a formulation. Sodium dodecyl sulphate (SDS)-based nanocarriers resulted in slightly improved cytotoxicity. Overall, the results highlight and envisages the usage of excipient in nano-formulations in a bid to improve chemosensitization of drug resistant cancer cells towards anti-cancer drugs.

3.2. Introduction

Among several efflux transporters, P-glycoprotein (P-gp), has garnered enormous attention in cancer research as well as drug delivery. It is a multidrug resistance protein of the ATP-binding cassette (ABC) family of efflux transporters[1]. ATP-dependent drug efflux leads to reduced effective cellular concentrations of anti-cancer drugs. In addition to being expressed in tumor cells, P-gp expression normally occurs in cells of kidney, breast, brain, colon, liver and pancreas. Consequently in cancer models of these tissues, their over-expression leads to MDR.

P-gp inhibitors developed over the years have proved disappointing in most of the clinical trials due to unpredictable pharmacokinetic interactions with co-administered anti-cancer drug and inherent inhibitor toxicity leading to toxic plasma concentrations and side effects in patients[2]. Nano-formulations carrying anti-cancer drugs, on the contrary, can revert drug resistance with minimal interference in the pharmacokinetic profile of the drug and a more localized action within tumor tissue. Different drug delivery systems such as nanoparticles (NPs) or liposomes etc., have shown promise in their ability to bypass P-gp mediated drug efflux, enhance cellular uptake, increase intracellular concentration of co-administered anti-cancer drug, exhibit minimal inherent toxicity compared to the inhibitors and act as a reservoir for both inhibitors and anti-cancer drugs for a sustained therapeutic outcome[3].

Several excipients routinely used in pharmaceutical industry as drug solubilizers and stabilizers in various formulations are known for their ability to reverse P-gp mediated drug efflux. These belong to classes as diverse as polymers (polyethylene glycol (PEG)), lipids (peceol), excipients/surfactants (Cremophor® EL, SDS) and are derived from natural or synthetic sources[4,5]. Non-ionic surfactants including Cremophor® EL, polysorbate 80 (Tween® 80) and Solutol® HS15 have been extensively evaluated[6] for their P-gp inhibitory ability in P-gp over-expressing Caco-2 cell monolayers[7] and everted gut sac model[8]. Recently, Cremophor® EL and Tween® 80 were shown to inhibit another significant MDR transporter- multidrug resistance protein (MRP2) more efficiently than P-gp[9] exhibiting dual P-gp-MRP2 inhibition capability.

Few studies are coming to fore, wherein excipient with MDR modulatory properties when incorporated in nanocarriers mediate drug resistance reversal in cancer. Lipid nanocapsules (LNC)[10,11] and solid lipid NP (SLN)[12] developed recently used Solutol® HS15 in their preparation.

Nanohybrid liposomes- incorporating Solutol® HS15, Pluronic® F68 and Cremophor® EL, were reported to show positive results in paclitaxel resistant lung carcinoma cell lines[13]. However, a comparative study with non-ionic, cationic and anionic surfactants incorporated polymeric NPs is lacking in this regard.

In this work, we conceptualize that when 'surface active agents' are incorporated in NP, they get immobilized on the surface of nanocarriers, behaving as P-gp efflux pump modulator as soon as they come in contact with tumor cell. This possibly enables bypassing/ modulating P-gp transporter proteins by different mechanisms on the cellular membrane[14].

Poly lactic-co-glycolic acid (PLGA) based polymeric NPs were prepared by solvent evaporation method with different surfactant combinations. In addition to non-ionic surfactants (Cremophor® EL, Solutol® HS15 and Tween® 80), SDS (anionic) and CTAB (cationic) were included in the present study to compare the effect of surface charge of nanocarrier on P-gp inhibition and the possible cell-nanocarrier interaction. Cell adhesion, P-gp inhibitory abilities and cytotoxicity of the particles co-administered with anti-cancer drug doxorubicin were evaluated and compared in this work.

3.3. Materials and methods

3.3.1. Materials

Polyvinyl alcohol (Mowiol 4-88) and PLGA resomer502H were kind gift from Kuraray (Frankfurt, Germany) and Evonik® (Darmstadt, Germany) respectively. SDS was obtained from Carl Roth (Karlsruhe, Germany) while Cremophor® EL and CTAB were provided by Fluka Analytical. Polyethylene glycol-660 hydroxystearate (PEG-HS, Solutol® HS15) and Tween® were obtained from BASF AG (Ludwigshafen, Germany) and Caelo (Hilden, Germany) respectively. Rhodamine-123, calcein acetoxymethylester (calcein AM), wheat germ agglutinin-FITC, doxorubicin hydrochloride, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT), nile red and all other solvents and reagents were purchased from Sigma Aldrich (Steinheim, Germany) and were of analytical grade. FITC-conjugated secondary antibody and glycoprotein-P monoclonal antibody (C219) were obtained from Biozol (Eching, Germany) and Thermo Scientific (Rockford, USA) respectively.

3.3.2. Cell lines

Glioma cell lines of F98 (glioblastoma) and 9L (gliosarcoma) were obtained from ATCC (Manassas, VA, USA). The cell-lines were passaged in T-75 tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 0.1 mg/mL streptomycin in a humidified 37°C incubator with 5% CO2. All cell culture grade chemicals were purchased from Sigma Aldrich. Passages between 5 and 30 were used for the experiments.

3.3.3. Preparation and characterization of nanoparticles

PLGA nanoparticles were prepared by solvent evaporation method using an o/w emulsion solvent evaporation process. The organic phase comprised of 20mg PLGA dissolved in 400µL ethyl acetate prior to all the preparation steps. The aqueous phase was prepared with 2mL of various surfactants- PVA, PVA with SDS, Cremophor® EL, CTAB, Solutol® HS15 and Tween® 80 at different concentrations (Table 1). The organic phase was emulsified with the aqueous phase (50% duty cycle for 4 min) by sonication using a microtip probe sonicator (Bandelin Sonoplus, Germany) in an ice bath. The organic mixture was then removed under reduced pressure for 20 min. This was followed by centrifugation at 12,000 rpm for 20 min at 6°C (Universal 320 R Hettich Cetrifuge, Tuttlingen Germany) to remove excess surfactants from NPs. Nile red particles (NR-NP) were similarly prepared by adding 40µL of 0.1% nile red solution in the organic phase. The nanoparticles were analyzed for their size distribution, polydispersity index (PDI) and zeta potential using a ZetaPlus (Brookhaven Instrument Corporation) (Table 1). Each sample was analyzed in triplicate.

3.3.4. P-gp expression analysis

For sample preparation of cells for flow cytometry, cells were trypsinized, counted and centrifuged. Aliquots of 0.5 million cells per sample were re-suspended in 50µL PBS and incubated with C219 primary antibody for 15 min in dark at 4°C. Cells were washed twice with FACS buffer (PBS, heat inactivated FCS (1%), sodium azide (0.09%)). Further incubation with

secondary antibody was done as aforementioned with primary antibody. Cells were washed again and re-suspended in FACS buffer. All measurements were made on a BD FACSCantoII (BD Biosciences, San Jose, CA) flow cytometer. Appropriate controls were used for both cell lines.

3.3.5. *In-vitro* cytotoxicity

Approximately 5,000 cells per well were seeded in 96-well plates and grown in DMEM overnight. Cells were then incubated with the different nanoparticle preparations of equivalent polymer concentrations (500- 0.5µg/mL) for 24 hr. After carefully removing the supernatant followed by two washing steps in PBS (pH 7.4), cells were further incubated with the MTT (5mg/mL). The cells were then lysed and MTT crystals dissolved in dimethyl sulfoxide. The plate was read at 544nm using a plate reader (Victor 3, Perkin Elmer). The cell viability was calculated using an untreated control as reference. For evaluating the chemosensitization of cells, both excipient-based NPs and their equivalent excipient concentrations in solution form were tested. MTT was performed together with increasing doxorubicin concentrations following same protocol at a particle concentration of 50µg/mL. Control experiments were performed with medium containing 10% (v/v) DMSO.

3.3.6. Nile red cell adhesion assay

5,000 cells were plated per well in 96-well plate and incubated overnight for adherence. After removing supernatant, treatment was given with NR-NPs encapsulating 0.5µg/mL equivalent nile red. After 4 and 24 hr incubation, cells were washed once with ice-cold PBS followed by addition of 100 µL ethanol solution per well. The absorbance was measured at (544 nm/ 615 nm) with a plate reader (Victor 3, Perkin Elmer).

3.3.7. Calcein AM accumulation assay

For determining the effect of nanoparticles on P-gp inhibition, a calcein AM accumulation assay was performed as described elsewhere[15] with small modifications. Following trypsization and centrifugation of glioma cells and repeated washing steps with Krebs-Hepes buffer (KHB), cells were seeded into black 96-well plates (Greiner, Frickenhausen, Germany) at a density of

approximately 20,000 cells per well to which 50µg/mL NP was added. After a 30 min pre-incubation period, 2.5 µM calcein AM solution was added to each well. The fluorescence was measured immediately in constant time intervals (60 s) up to 240 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm with a BMG POLARstar microplate reader maintained at 37°C.

3.3.8. Rhodamine-123 uptake assay

To re-evaluate the effect of inhibitors on P-gp, a rhodamine-123 accumulation assay was performed as it is a known P-gp substrate. 5,000 cells each of F98 and 9L were seeded in 96-well plates and incubated overnight for adherence. Rhodamine-123 (0.3µM) together with 50µg/mL NP treatment was given. Following incubation for 1 and 4hr, media was removed, and wells washed once with PBS. 100µL Triton-X 100 (1% v/v) was added to each well and incubated for 30min to lyse cells and extract rhodamine-123. The fluorescence was measured at an excitation wavelength of 505 nm and an emission wavelength of 540 nm with a fluorescence spectrophotometer (Victor3, Perkin Elmer) and normalized for cellular protein levels as determined by bicinchoninic acid (BCA) assay.

3.3.9. Statistical Analysis

All the experiments were performed in triplicates and reported as mean ± S.D. Statistical comparisons were made with one-way ANOVA followed by Dunnett's post test using GraphPad Prism software version 5.0. In all cases, $p<0.05$ was considered to be significant at 95% confidence level.

3.4. Results

3.4.1. Physicochemical characterization

Table 1 shows the respective surfactant concentration used for particle preparation and their corresponding size, polydispersity indices (PDI) and zeta potential. PVA NPs (115 ± 15 nm) had smallest size of all NPs and there was a slight increase in particle size on addition of anionic surfactant SDS in PVA+SDS NPs (133 ± 8 nm). This could be due to the self-assembling property of anionic surfactants being altered by the non-ionic surfactant PVA forming more stable NPs.

On the other hand, the addition of cationic and non-ionic surfactants with long hydrophilic chains led to bigger particles. Solutol® HS15 NPs exhibited largest size (267 ± 7 nm) which was accompanied by a slight increase in PDI value (0.16).

Zeta potential of particles varied clearly showing the effect of surfactant on surface properties of nanocarriers. CTAB NP's demonstrated a cationic character while the other surfactants exhibited negative surface charges which could be explained due to their inherent charge properties and the use of anionic PLGA as the polymer.

Two sets of cytotoxicity assays were performed. To assess the toxicity exhibited by different NPs against gliomas, MTT assays were performed for 24 hours with varying NP concentrations. Results were reported as LC_{50} (lethal concentration, 50%) of NPs (Table 1). Except CTAB NPs, none of the formulations were cytotoxic up to 500µg/mL NP concentration. CTAB particles showed slightly higher toxicity (LC_{50}- 199 ± 80.3µg/mL) in F98 cells than the more resistant 9L cells (LC_{50}- 312 ± 24µg/mL). In all subsequent *in vitro* assays a NP concentration of 50µg/mL was used. MTT results with doxorubicin are explained in section 3.6.

	Concentration (g/100mL)	Size (nm)	Zeta potential (mV)	PDI	LC_{50} (µg/mL)	
					F98	9L
PVA	1	115.07 (± 15.55)	-13.07 (± 0.98)	0.10 (± 0.03)	>500	>500
SDS+PVA	0.05	133.43 (± 8.55)	-17.27 (± 4.48)	0.10 (± 0.04)	>500	>500
Cremophor® EL	0.2	224.6 (± 2.25)	-13.35 (± 0.99)	0.10 (± 0.01)	>500	>500
CTAB	0.02	239.93 (± 1.16)	6.02 (± 2.61)	0.11 (± 0.04)	199 (± 80.3)	314 (± 24)
Solutol® HS 15	0.1	267.57 (± 7.54)	-13.26 (± 2.15)	0.16 (± 0.02)	>500	>500
Tween® 80	1	180 (± 2.96)	-18.24 (± 0.78)	0.11 (± 0.00)	>500	>500

Table 1: Physicochemical characterization of nanoparticles prepared with different surfactants. The hydrodynamic diameter, polydispersity index (PDI) and zeta potential was analyzed by PCS. LC_{50} values of all NP formulations following their incubation with F98 and 9L cells for 24h. (Mean ± SD; n=6).

3.4.2. Protein expression analysis

Gliomas were used in the current study as they represent the most malicious and aggressive cancers of all[16]. To demonstrate the magnitude of expression of P-glycoprotein, P-glycoprotein surface expression was examined in both F98 and 9L cells using the monoclonal antibody C219 which recognizes the COOH-terminal cytoplasmic sequence of P-glycoprotein isoforms. Although F98 cells are known to over-express P-gp, in 9L cells, their expression was found to be more than in F98 (Fig.1) ascribing an even higher resistant character to 9L cell lines than F98.

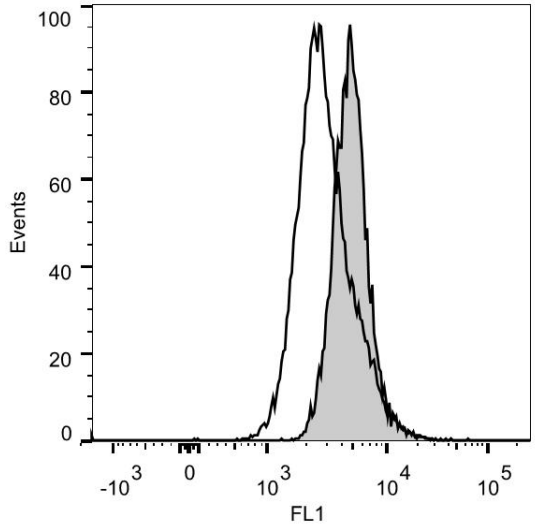

Figure 1. Histogram of P-gp expression in F98 glioblastoma (white) and 9L gliosarcoma (grey) cell lines.

3.4.3. Cell adhesion studies

To understand the interaction between cells and particles, NR-NP with all surfactants were prepared and cells treated for 4 and 24hr. These time points were chosen because *in-vitro* accumulation and cytotoxicity assays were performed within these time-frames respectively. At 4hr, only Cremophor® EL NR-NP demonstrated significant adherence in comparison with free nile red solution in both cells (Figure 2a). 24hr post-treatment showed slightly higher adherence of NR-NPs in both cell lines than 4hr treatment (Figure 2b). However, this increase was significant with Cremophor® EL and CTAB NR-NPs in both cell lines and PVS+SDS nile red NPs in F98 cell line. Thus, the interaction between NPs and cells appeared to be surfactant and cell type-dependent.

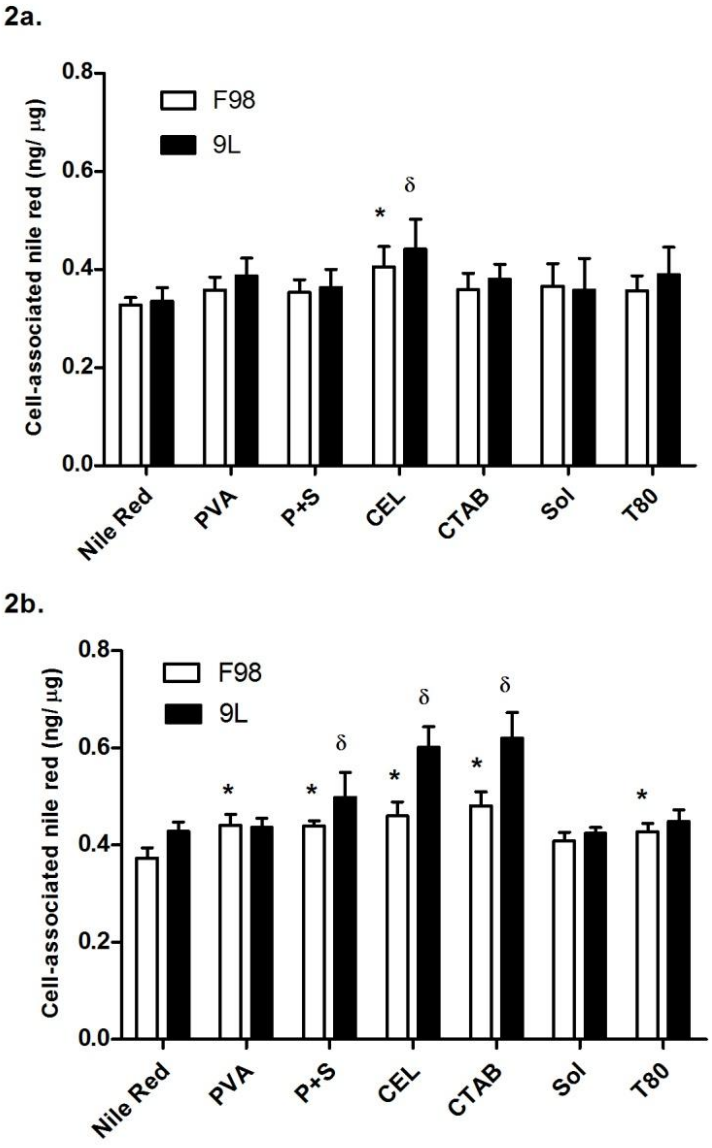

Fig. 2. Cell-associated particles after incubation with F98 and 9L cells for 4hr and 24hr at a concentration of 0.5µg/mL equivalent nile red. The interaction was quantified by determination of fluorescence intensity following nile red extraction with ethanol. (Mean ± SD; n=6). One-way ANOVA followed by Dunnett's post test; Fig.2a. 4hr *$p < 0.01$ and $^{\delta}p < 0.01$ compared with nile red solution. Fig. 2b. 24hr *$p < 0.01$ and $^{\delta}p < 0.01$ compared with nile red solution.

3.4.4. Calcein AM assay

The ability of NPs to modulate P-gp function was evaluated using calcein AM assay in both P-gp expressing cells. Following diffusion, calcein AM gets hydrolyzed by esterases present in cells to

calcein anion which is fluorescent and hydrophilic. In P-gp over-expressing cells, increased fluorescence of intracellular calcein is measured as a function of P-gp inhibition. The results (Figure 3) represent rate of accumulation of calcein within cells. As can be observed, the results showed a significant rate of calcein accumulation in Cremophor® EL and CTAB NPs in both (9L and F98) and PVA+SDS NPs in F98 cell lines in comparison to control (C-AM without any treatment). The decrease in calcein level in Solutol® HS15 treated NPs was an exception as the accumulation rate was much slower than with free calcein levels. One possible explanation could be the entrapment of the fluorescent dye into the micelles formed following the detachment of loosely bound Solutol® HS15 molecules on NP surface. These micelles tend to get entrapped in the membrane resulting in loss of interaction with the P-gp transporter protein[9,13].

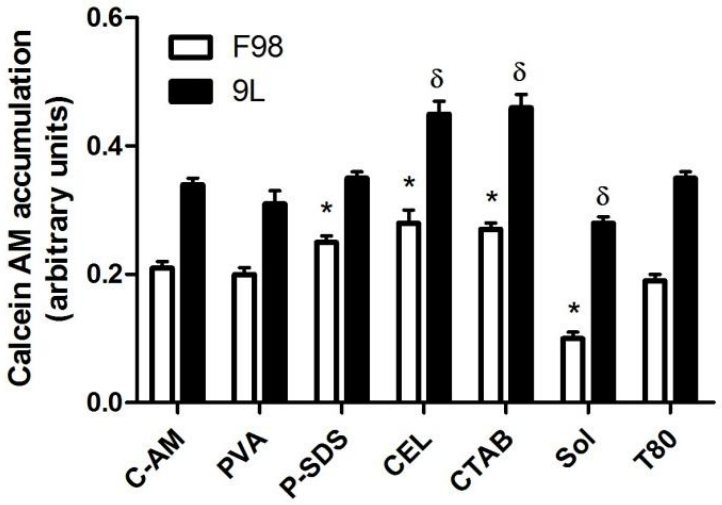

Figure 3. Calcein AM accumulation assay after incubation with particles (50µg/mL NP suspension) for 3hr. (Mean ± SD; n=6). ; *$p < 0.05$ and $^{\delta}p < 0.05$ compared to calcein AM accumulation (without NP) in F98 and 9L cells respectively, One-way ANOVA followed by Dunnet's post test.

3.4.5. Rhodamine-123 uptake assay

Rhodamine-123, a P-gp substrate and fluorescent dye is routinely used to study the MDR phenomenon. Rhodamine-123 uptake increased in all treatment groups including control group (rhodamine-123 solution) and was markedly enhanced after the 4hr treatment in comparison

to the 1hr treatment (Figure 3). Excipient-based NPs appeared to facilitate the dye uptake by sensitizing the efflux transporter function in both resistant cell lines. Accumulation was significantly high in CTAB, Cremophor® EL and PVA+SDS NPs in both cell lines by 4 hours. In F98 cells, which are comparatively less resistant, almost all particle preparations sensitized the cells leading to enhanced substrate accumulation except for Solutol® HS 15 NPs. Rhodamine-123 binds strongly (Kd=2µM) to serum proteins present in cell culture media[17]; consequently, as the uptake experiments were done in serum-free media, a 24-hour study was not feasible.

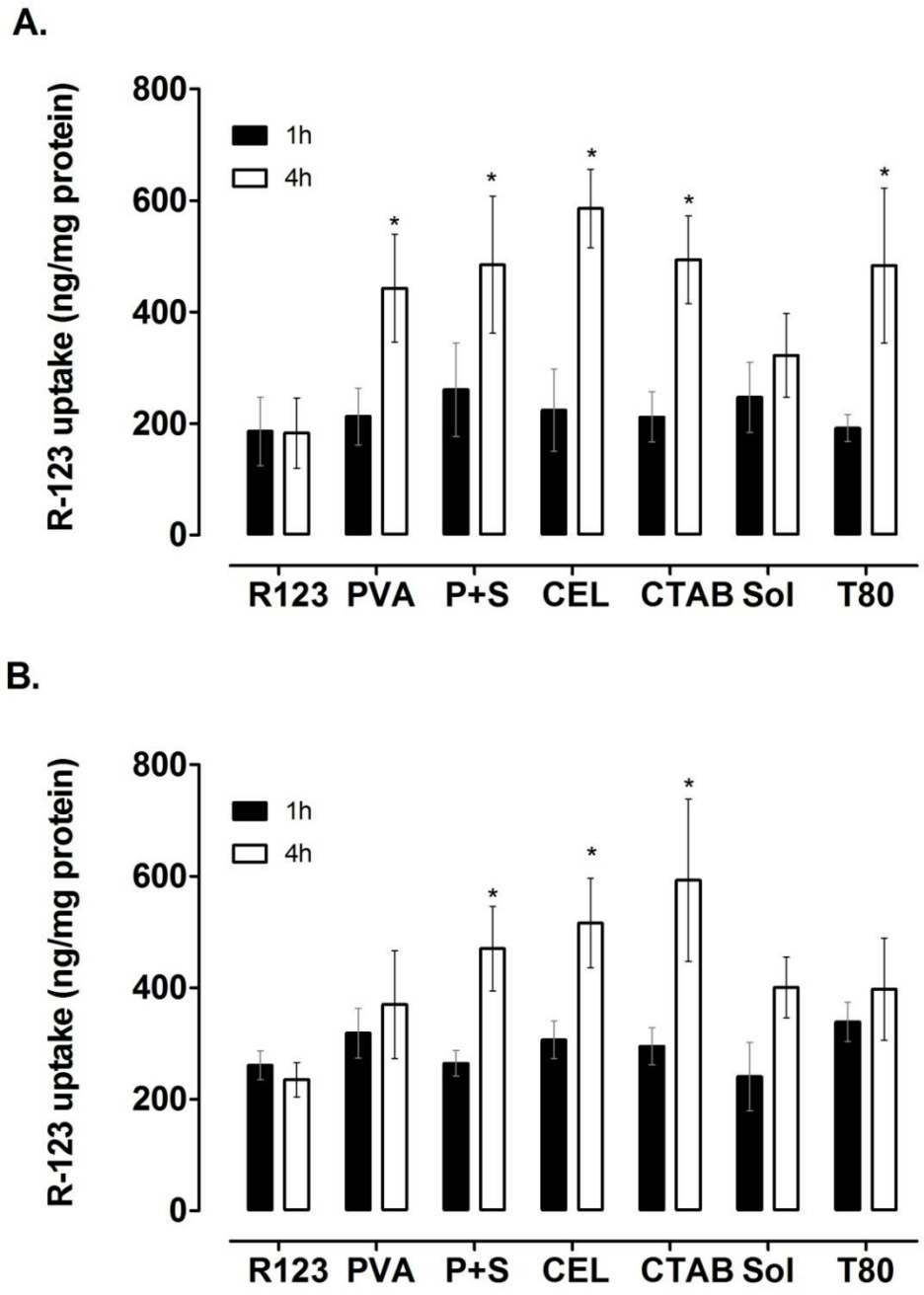

Figure 4. Rhodamine-123 accumulation was evaluated following treatment with NP's prepared with different surfactants for 1 and 4hr in A. F98 and B. 9L glioma cells. (Mean ± SD; n=9). *p<0.001and $^{\#}$p<0.001 compared to rhodamine-123 accumulation (without NP) in both cells, One-way ANOVA followed by Bonferroni Multiple comparison post test.

3.4.6. Cytotoxicity studies

As seen from MTT assay results (Figure 5), gliosarcoma 9L cell line appeared to be less sensitive to doxorubicin than F98. Following 24hr chemosensitization of cells with different NP formulations and their corresponding excipients, Cremophor® EL and CTAB appeared to modulate MDR transporter function the most. As compared to control (treatment with doxorubicin), Cremophor EL and CTAB treatment (both NPs and their equivalent surfactant dose) resulted in lower IC_{50} values (Table 2).

PVA and Solutol® HS15-based NPs did not appear to show any significant reversal although free Solutol® HS15 led to approximately 3-fold lower IC_{50} values in both cell line. In substrate accumulation experiments, similar observations were made. In comparison to doxorubicin and SDS treated group, PVA+SDS NPs showed lower IC_{50} in both cells which was an extrapolation of previous results as well. In general, NPs (Figure 5. white bars) exhibited higher IC_{50} values than their corresponding excipient component (Figure 5. black bars).

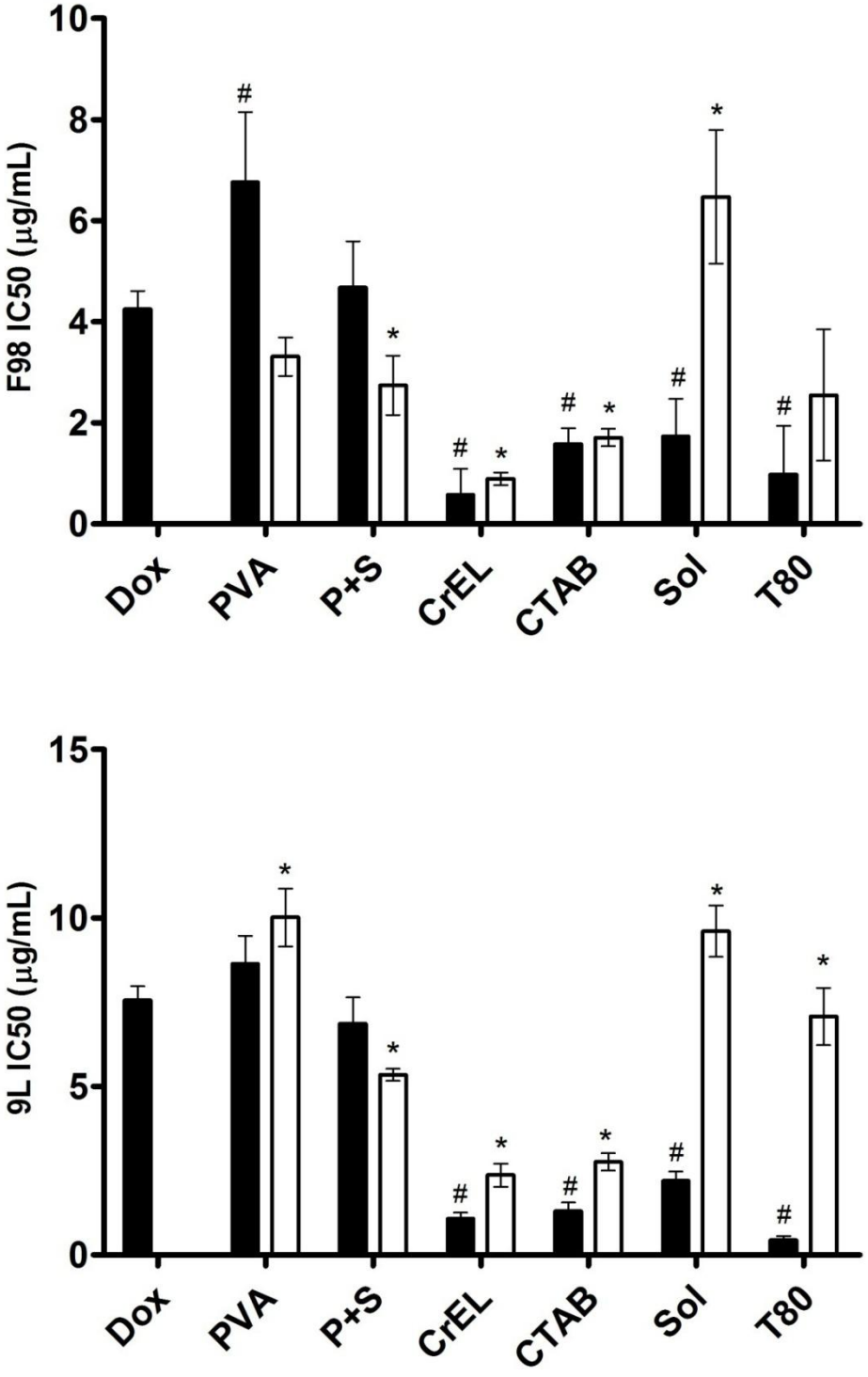

Figure 5. IC_{50} values of doxorubicin in F98 and 9L cell lines following NP treatment (white bars) and equivalent excipient concentrations (black bars) for 24h (Mean ± SD; n=3). *p< 0.05 and #p< 0.05 compared with doxorubicin solution.

3.5. Discussion

FDA approved excipients/ surfactants have found regular use as coatings and solubility enhancers in pharmaceutical industry. Many excipients, though not inherent MDR inhibitors, have shown P-gp modulatory function and sensitize resistant cells to MDR substrates. In this study, non-ionic and ionic nanoparticles were developed and assessed in highly resistant glioma cell lines for their ability to overcome MDR.

The effect of various process parameters (surfactant concentration, power and duration of sonication) on the feasibility of stable nanoparticle were studied. The stable formulations used in this study exhibited different surface properties. The particles ranging 100-300 nm in size and surface properties were reflective of the different types of 'surface active agent' and their concentration (Table 1). Except CTAB-based cationic NPs, all formulations showed negative surface charge and narrow polydispersity index. Amongst all the nanocarriers prepared, CTAB NPs showed comparatively higher toxicity in both cell lines. Once the cytocompatibilities of NPs were established, all subsequent *in vitro* studies were performed at a considerably lower concentration of 50µg/mL equivalent polymer concentration (approximately 5µL/mL NP suspension).

Nile-red cell adhesion studies were performed in order to understand the dynamics of NP adhesion to the cell surface. Detergents and excipients tend to disrupt the P-gp-lipid interface affecting functional conformation of efflux transporters. Cell-NP interaction showed significantly higher accumulation of nile-red in both cell lines treated with Cremophor® EL, PVA+SDS and CTAB NPs post 24hr treatment as compared to an equivalent concentration of nile red solution. The results imply an enhanced cell-NP interaction function following NP treatment.

Accumulation studies with both P-gp substrates- calcein AM and rhodamine-123, revealed an increasing pattern after approximately 4hour post treatment in the following order- CTAB NP> Cremophor® EL NP> PVA+SDS NP. PVA and Tween® 80 NPs were found to be moderately effective in overcoming P-gp mediated resistance. Solutol® HS15 NPs showed anomalous behavior and resulted in a decreased uptake of calcein compared to that of control group. Using rhodamine-123 as P-gp substrate, Solutol® HS15 NP treatment group did not show any

significant improvement of P-gp modulatory function. Free Solutol® HS15 units released from NP could have trapped hydrophobic rhodamine-123 in micelles which may ultimately get blocked in the rigid membranes of drug resistant cell, thereby reducing the substrate uptake. This has been observed in previous studies and is responsible for a lower treatment efficacy[9,13].

9L cells have been demonstrated to possess a set of extremely chemoresistant cancer stem-like cells (CSLCs)[18]. High density of P-gp efflux transporters (Figure 1) on cell membrane of 9L cells could be responsible for their higher resistance against doxorubicin (Table 2). It has been reported that doxorubicin failed to eradicate cancer stem cell populations from anaplastic thyroid carcinoma cells[19].

Solutol® HS15 based NPs failed to show therapeutic effects in all assays. Except Solutol® HS15 NPs, all other formulations showed enhanced uptake of doxorubicin by F98 cells with a lower IC_{50} values of up to 4.8- fold for Cremophor® EL and 2.5-fold for CTAB NPs. 9L cells showed around 3-fold reduced IC_{50} values with these two set of NPs, as compared to doxorubicin-treated cells (control).

NPs led to a decrease in cytotoxicity towards doxorubicin compared to free excipients in general, which might be due to the fact that during NP preparation, unbound excipients molecules may have been washed. Another explanation might be that above critical micellar concentrations (CMC), certain excipients as Solutol® HS15 (CMC= 0.02% w/v) and Tween® 80 (CMC= 0.0016% w/v), can form micelles in free form which can lead to reversal of drug resistance due to their interaction with cell membrane.

Thus, in the context of nanoparticle formulations prepared using different surfactants, Cremophor® EL and CTAB based NPs, and to some extent PVA+SDS NPs appear to reverse MDR in this study. Cremophor® EL has demonstrated its superiority amongst other excipients on the absorption of P-gp substrates *in vitro* and *in vivo* in previous studies[8,20]. CTAB particles on the other hand, are known to bind to the negative regions of the cell membrane followed by their rapid internalization[21] in studies that have previously shown their MDR modulatory ability in cancer[22,23].

Recently, liposomes inserted with non-ionic surfactants such as Solutol® HS 15, Pluronic® F68 and Cremophor® EL were evaluated in P-gp expressing A549/T cells for reversing MDR[13].

Amongst the three formulations, reduced P-gp expression levels and ATP depletion were reported with Solutol® HS15 incorporated liposomes in comparison to Cremophor® EL liposomes. In this study, the initial concentration of surfactants used for liposome preparation was the same and Solutol® H15 scored better than Cremophor® EL in MDR reversal. However, this contradictory outcome in our work could be explained on the basis of different concentration of surfactants that were used: Solutol® HS15-0.1% w/v and Cremophor® EL-0.2% w/v; and also on the basis of different formulation types in both studies.

With respect to structural attributes, all non-ionic surfactants used in this study have polyethylene glycol (PEG) chains. In comparison to Solutol® HS 15 (PEG15) and Tween® 80 (PEG20), Cremophor® EL (PEG35) has longer chain. Hydrophilic chains interact with the polar head groups of the plasma membrane while the lipophilic head group of surfactant interacts with the lipid bilayer[6]. The long PEG chain in case of Cremophor® EL, causes stronger steric-repulsion from the polar head groups of plasma membrane making the excipient less toxic than the other two surfactants[24]. Although this does not explain the interaction of excipient with the P-gp transporter, the mode of action of free excipient versus nanocarriers developed with same excipient may understandably differ. An in-depth and comparative study of interaction pattern between excipient-lipid bilayer and excipient-based NP-lipid bilayer can provide a better understanding of the P-gp inhibition step. As the particles are localized within the tumor mass, the risk of excipient exposure elsewhere in the body can be presumed to be minimal. Further elaborate *in vivo* studies can help to understand the pharmacokinetics of excipient-based NPs. Once, these interactions are chalked out, it would be interesting to manoeuvre different ‘surface active‘ MDR modulators and utilize them in preparing chemosensitizing drug delivery systems.

The concentrations at which these excipients mediate P-gp modulation are much lower than used in clinical applications for drug delivery. Hence, surfactant-based formulations can offer promising alternatives compared to regular nanocarriers with their ability to 1) deliver anti-cancer payload to the tumor tissue, 2) sensitize drug resistant cells towards anti-cancer drugs, 3) improve unfavourable pharmacokinetic side effects associated with drug-clearance, and 4)

deliver an MDR inhibitor which can not only act as a 'dual strategy' for reversing MDR, but also reduce the systemic toxicity due to high doses of commercial inhibitors.

3.6. Conclusion

Amogst the ionic and non-ionic surfactants tested, Cremophor® EL NP and CTAB NP showed most efficient MDR reversal activities when co-administered with doxorubicin in highly resistant glioma cell lines. Moderate effects were also observed with anionic- SDS NPs in the less resistant cell line. More than just carrying anti-cancer drug payload and P-gp inhibitors, these nanocarrier systems can circumvent MDR by evading drug efflux transporters. Further studies aimed at understanding NP-cell interactions would facilitate the development of these specific 'surface active' nanoformulations. Such 'surface active' drug delivery systems appear promising and can well be considered as the fourth generation of MDR inhibitors for the treatment of clinical MDR in cancer.

3.7. References

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4. Chapter 2:

Cargoing P-gp inhibitors via nanoparticle sensitizes tumor cells against doxorubicin

4.1. Abstract

Inhibitors against multidrug resistance (MDR) efflux transporters have failed in most clinical settings due to unfavorable pharmacokinetic interactions with co-administered anti-cancer drug and their inherent toxicities. Nanoparticles (NPs) have shown potential to overcome drug efflux by delivering and localizing therapeutic molecules within tumor mass. In this work, we investigated effect of nanocarrier surface charge and formulation parameters for a hydrophilic and lipophilic MDR inhibitor on their ability to reverse drug resistance. Active inhibition of efflux pumps was achieved by encapsulating first and third generation P-gp inhibitors- verapamil and elacridar respectively in non-ionic, anionic and cationic surfactant-based NPs. The ability of NPs to reverse P-glycoprotein (P-gp)-mediated MDR efflux was evaluated in sensitive (A2780) and resistant (A2780Adr) ovarian cancer cell lines by various *in vitro* accumulation and cytotoxicity assays. Uptake mechanism for NP appears to be caveolae-dependent with 20%-higher internalization in A2780Adr than A2780 cell lines which can be co-related to the biophysical membrane composition. Cationic- CTAB NPs showed highest reversal efficacy followed by PVA and SDS-NP (P+S-NP) and PVA-NPs. As compared to doxorubicin treated drug resistant cells lines, blank-, verapamil- and elacridar-CTAB-NPs showed 2.6-, 20- and 193-fold lower IC_{50} values. This work highlights the importance of inhibitor-loaded charged particles to overcome cancer drug resistance.

4.2. Introduction

Members of the ATP-binding cassette (ABC) superfamily of transmembrane transporters such as P-gp, multidrug resistance protein (MRP) and breast cancer resistance protein (BCRP) are expressed at various physiological barriers (gastrointestinal tract, lung, kidney, blood-brain barrier) and play protective roles against xenobiotics and in drug metabolism and excretion from the body. An over-expression of one or more of these transporters in tumor cells leads to recognition and systematic efflux of anti-cancer drugs and their structural analogues, clinically referred to as the multidrug resistance (MDR) in cancer.

MDR is the cause of failure of most chemotherapeutic regimens in cancer treatment and also for cancer relapse. Treatment regimens employing proper P-gp inhibitor and P-gp substrate combination can check development of cancer drug resistance by potentiating synergistic actions. Different generations of pharmacological MDR inhibitors have been researched in last decades but most drug trials were disappointing as the inhibitors lack specificity and exhibit high systemic toxicity amongst other reasons[1]. Other factors such as the limited solubility of P-gp inhibitors in aqueous solution and low availability at the tumor site have also contributed to the failure of P-gp inhibitors in overcoming MDR in cancer.

Drug delivery systems offer promising alternative for transportation of an array of molecules enabling anti-cancer drug uptake in cancer cells[2]. In most of the studies done so far, a combination of anti-cancer drug and/ or P-gp inhibitor were co-administered in a multitude of carriers (lipid nanocapsules[3], liposomes[4], microspheres[5]) to sensitize drug resistant cells to chemotherapy. As there are already many market approved nano-based anticancer drugs such as Doxil® or Abraxane®, focus should be diverted towards the safe and localized delivery of MDR-inhibitors to the tumor mass. This is of urgent attention due to the fact that the ABC transporters are constitutively expressed in different barrier tissues and administration of MDR inhibitors as a formulation reduces the risk of systemic exposure and interference in routine functioning of these transporter proteins. Moreover, an optimal concentration of MDR inhibitors can be attained locally at the tumor site due to enhanced permeability and retention (EPR) effect.

To this effect, only Binkhathlan and colleagues have done an extensive *in vivo* investigation using valspodar-loaded methoxy-poly(ethylene oxide)-block-poly(e-caprolactone) (PEO-b-PCL) micelles[6]. Use of micellar drug delivery systems, not only improved valspodar solubility but reduced its pharmacokinetic interactions with co-administered doxorubicin solution in Sprague-Dawley rats. Thus, nanocarriers are efficient in reducing the effective dose of P-gp modulator at which they attenuate P-gp-mediated efflux activity and improve their bioavailability at the pharmacological site of action.

Drug resistant cells have highly rigid and compact cellular membrane due to altered phospholipid profiles[7]. As nanoparticle surface is the first to interact with lipid bilayer, it is important to characterize charge-dependent sensitization of drug resistant cells. In our previous work, nanoparticles with different 'surface active agents' were evaluated for their ability to overcome MDR [Chapter 2]. In order to obtain complete reversal and evaluate the effect of particle charge, inhibitor-loaded NPs were developed with non-ionic (polyvinyl alcohol (PVA)), cationic (cetyltrimethylammonium bromide (CTAB) and anionic (sodium dodecyl sulphate (SDS) surfactants. P-gp inhibitors- verapamil hydrochloride (VRP) and elacridar were chosen due to their different solubility profiles, were loaded in aforementioned NPs and tested in present work. To compare the quantitative efficacy, we used P-gp expressing, adriamycin resistant human ovarian cancer cell line A2780Adr and its parental cell line A2780 as control.

4.3. Materials and Methods

4.3.1. Materials

Poly-lactide-co-glycolide (PLGA) RG 502 H was obtained from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA, Mowiol® 4-88) and verapamil hydrochloride were kind gift from Kuraray (Frankfurt, Germany) and Fagron (Barsbüttel, Germany) respectively. Sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) were obtained from Carl Roth (Karlsruhe, Germany) and Fluka Analytical respectively. Elacridar (CAS: 143664-11-3) was synthesized in-house with a purity of > 98%. Rhodamine-123, calcein AM, doxorubicin, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT), nile red, wheat germ

agglutinin-FITC, DAPI and all other solvents and reagents were obtained from Sigma Aldrich (Steinheim, Germany) and were of analytical grade.

4.3.2. Cell lines

The human ovarian carcinoma cell line A2780 and the corresponding P-gp over-expressing adriamycin resistant A2780Adr cell line (ECACC Nos. 93112519 and 93112520)were kind gift from Prof. Dr. Michael Wiese, Department of Pharmaceutical Chemistry II, University of Bonn. The cell lines were grown in RPMI-1640 medium supplemented with 10% FBS, 50µL/mL streptomycin, 50 U/mL penicillin G, and 2 mM L-glutamine. All cell lines were cultivated in a 37 °C incubator with 5% CO_2/ 95% humidified air. Subculturing was performed after confluence of 80-90%, using 0.05% trypsin and 0.02% EDTA. All cell culture grade chemicals were purchased from Sigma Aldrich.

4.3.3. Preparation and characterization of nanoparticles

Internal phase was prepared by dissolving 20 mg PLGA in 400 µl dichloromethane. This was poured in 2 mL of aqueous phase (1% PVA/ 1% PVA and 0.05% SDS/ 0.02% CTAB solution) and ultrasonicated (50% duty cycle for 30sec). The organic phase of internal phase was evaporated by stirring for 3hr. 4 mg VRP or 0.4 mg elacridar were added to internal phase to prepare verapamil (VNP) or elacridar NPs (ENP). Blank nanoparticles were prepared analogously without the addition of either inhibitor. With three surfactants (PVA/ SDS/ CTAB) and two generations of P-gp inhibitors- VRP and elacridar, nine different formulations were prepared (Table 1). Nile red NPs were similarly prepared by adding 40µl of 0.1% nile red solution in the organic phase and contained no inhibitors. Nanoparticles were analyzed for their size distribution, polydispersity index (PDI) and zeta potential using a ZetaPlus (Brookhaven Instruments Corporation). Each sample was analyzed in triplicate.

4.3.4. Encapsulation efficiency and inhibitor release

The encapsulation rate was analyzed indirectly by determining the amount of non-encapsulated drug in the external aqueous phase by high-performance liquid chromatography (HPLC). For determining the encapsulation efficiency of VRP following parameters were used: mobile

phase- KH_2PO_4 buffer (pH 3.0 adjusted with orthophosphoric acid): acetonitrile (60:40), flow rate 1.0 mL/min, wavelength 278 nm, LiChrospher® 60 RP-Select-B column (Merck, Germany) using a Waters 2695 with Photodiode Array Detector Waters 996 (Eschborn Germany).The amount of elacridar encapsulated within the NPs was detected using methanol: water (85:15), flow rate 1.5 mL/min, wavelength 254, Eurospher II 100-5 C18H (50 x 4 mm) column connected with a pre-column Vertex-Plus Eurospher II 100-5 C18H (5 x 4 mm) (Knauer, Germany). Samples were prepared in triplicate and encapsulation yield (%) was determined by dividing the experimental drug payload by the theoretical payload.

In order to assess the drug release, 2 mL of each NP preparation was taken in triplicate and kept at 37° C in a shaking water bath. Phosphate buffered saline (pH 7.4) was chosen as release buffer with 0.1 % Tween® 80 to maintain sink conditions. At predefined time points 0.5 mL of release medium was withdrawn and replaced by 0.5 mL of fresh buffer for continuous release. The amount of drug in the release medium was quantified for the respective MDR inhibitor by HPLC as mentioned before.

4.3.5. Confocal Laser Scanning Microscopy

Cells were grown over coverslips in 24 well plate and incubated with nile red nanoparticles for 4hr. Free nile red was removed by several washing steps followed by incubation with wheat germ agglutinin-FITC at 4°C for 30min. Fixation was performed with paraformaldehyde (4%) followed by nuclear staining with DAPI (300nM; 5min). Slides were prepared with glycerol/PBS and imaged with a Biorad Laser Scanning Confocal Imaging System.

4.3.6. Calcein AM accumulation assay

For determining the effect of encapsulated inhibitors on P-gp,a calcein AM accumulation assay was performed as described earlier (Chapter 2).Following trypsization and centrifugation of A2780 and A2780Adr cells and repeated washing steps with Krebs-Hepes buffer (KHB) cells were seeded into black 96 well plates (Greiner, Frickenhausen, Germany) at a density of approximately 20,000 cells per well to which 2.5 and 0.25 µg/mL of inhibitors VRP and elacridar and their NP equivalent were added. After a 30 min pre-incubation period, 2.5 µM calcein AM

solution was added to each well. The fluorescence was measured immediately in constant time intervals (60s) up to 240 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm with a BMG POLARstarmicroplate reader maintained at 37°C.

4.3.7. Rhodamine-123 uptake assay

To re-evaluate the effect of inhibitors on P-gp, a rhodamine-123 accumulation assay was performed as it is a known P-gp substrate. 10,000 cells each of A2780Adr and A2780 were seeded in 96 well plates and incubated overnight for adherence. Rhodamine-123 (0.3 µM) together with inhibitor (similar concentrations as in calcein AM assay) and equivalent NP treatment were administered in serum-free media. Following incubation for 6 hrs, media was removed, and wells washed once with PBS. 100µl Triton-X 100 (1% v/v) was added to each well and incubated for 30 min to lyse cells and extract rhodamine-123. The fluorescence was measured (ex = 505 nm, em = 540 nm) with a fluorescence spectrophotometer (Victor3, Perkin Elmer) and normalized for cellular protein levels as determined by bicinchoninic acid (BCA) assay.

4.3.8. *In vitro* cytotoxicity assay

In order to determine the toxicity of different formulations, cells were incubated with different blank (PVA, P+S and CTAB) nanoparticle preparations of equivalent polymer concentrations (500- 0.1µg/mL) for 24 hr. The sensitive counterparts of these cells were used as controls. Approximately 5,000 cells per well were seeded in 96 well plates and grown in DMEM overnight. Control experiments were performed with medium containing 10% (v/v) DMSO. After an incubation period of 72hr MTT was added (5mg/mL) and further incubated for 1hr. Formazan crystals formed were solubilized by adding 100 µl DMSO per well and measured photometrically at 544 nm using a plate reader (Victor3, Perkin Elmer). The cell viability was calculated using an untreated control as reference. To check the effectiveness of encapsulated inhibitors to reverse the drug resistance in P-gp (A2780Adr) over-expressing cells to doxorubicin MTT cytotoxicity assays were performed. Cells were treated with doxorubicin at different concentrations in a volume of 10µL. 2.5 and 0.25µg/mL of inhibitors VRP and elacridar and their NP equivalent were added to each well to achieve the final volume of 100µl.

4.3.9. Mechanism of nanoparticle endocytosis

Approximately 25,000 cells were seeded in 96-well plates and grown overnight for adherence. Cells were pre-incubated for 30 min with different endocytosis inhibitors at following concentrations: chlorpromazine hydrochloride- 10µg/mL[8], nystatin- 0.069mg/mL[9], methyl-β-cyclodextrin (MβCD)- 10mmol/L[10], 5-(N,N-dimethyl)amiloride hydrochloride (DMA)- 0.44mg/mL[9], Cytochalasin D- 10µg/mL[8] (all from Sigma) in HBSS serum-free media. Subsequently, nile red NPs were added and incubation further continued for 2 hr. After washing with PBS, 100µL isopropanol was added and the absorbance of nile red measured at (544nm/ 615nm) with a plate reader (Victor 3, Perkin Elmer). Energy dependence experiments were performed similarly by pre-incubating the cells at 4°C for 30 min prior to exposure to NPs. Nile red NP treated cells without inhibitors was used as control.

4.3.10. Statistical Analysis

All the experiments were performed in triplicates and reported as mean ± S.D. Statistical comparisons were made with one-way ANOVA followed by Dunnett's post test using GraphPad Prism software version 5.0. In all cases, $p < 0.05$ was considered to be significant at 95% confidence level.

4.4. Results

4.4.1. Physicochemical characterization

Physicochemical properties of blank and inhibitor (VRP and elacridar) loaded formulations are shown in Table 1. PVA and P+S NPs were comparatively larger in size than CTAB NPs within each subgroup. Inhibitor loading led to an increase in size of all NP's compared to blank particles. All formulations exhibited narrow size range as evident from polydispersity index (PDI). Zeta potential was strongly affected by the surfactant used and exhibited strongest negative and positive surface charge with anionic (SDS) and cationic (CTAB) surfactant respectively.

Two sets of cytotoxicity assays were performed. To assess toxicity exhibited by different NPs against both resistant and sensitive cell lines, MTT assays were done for 24hr with varying NP

concentrations. Except CTAB NPs, none of the formulations were cytotoxic up to 500µg/mL NP concentration (A2780Adr: LC_{50}-275.4±70.5µg/mL, A2780: LC_{50}-209±15µg/mL). In all subsequent *in vitro* assays an NP concentration of less than 10µg/mL was used. MTT results with doxorubicin are mentioned in section 3.6.

	Size (nm)	PDI	Zeta potential (mV)	Encapsulation (%) Verapamil/ Elacridar
Blank-PVA NP (B-PVA)	230.1 (± 1.8)	0.167 (± 0.08)	-12.59 (± 1.8)	-
Blank-PVA+SDS NP (B-P+S)	247.7 (± 9.9)	0.128 (± 0.01)	-18.29 (± 2.33)	-
Blank-CTAB NP (B-CTAB)	187.4 (± 14.2)	0.178 (± 0.05)	5.49 (± 1.58)	-
Verapamil-PVA NP (V-PVA)	292.9 (± 3.7)	0.106 (± 0.03)	-12.96 (± 0.83)	26.7 (± 1.4)
Verapamil- PVA+SDS NP (V-P+S)	312.2 (± 5.4)	0.066 (± 0.03)	-18.45 (± 0.84)	47.9 (± 0.2)
Verapamil-CTAB NP (V-CTAB)	195.4 (± 1.1)	0.169 (± 0.03)	6.52 (± 0.95)	11.8 (± 0.6)
Elacridar- PVA NP (E-PVA)	356.5 (± 9.2)	0.051 (± 0.08)	-14.57 (± 1.62)	89.3 (± 8.3)
Elacridar- PVA+SDS NP (E-P+S)	369.4 (± 9.4)	0.087 (± 0.03)	-18.67 (± 1.78)	95.8 (± 5.2)
Elacridar-CTAB NP (E-CTAB)	223.3 (± 45.2)	0.16 (± 0.01)	5.79 (± 0.85)	84.3 (± 8.8)

Table 1. Physicochemical characterization of NP formulations encapsulating verapamil/ elacridar; or without inhibitor (blank NPs). Size, polydispersity index (PDI) and zeta potential was measured by PCS. Encapsulation efficiency was calculated based on the theoretical content of inhibitors. (Mean± SD; n=3).

4.4.2. Encapsulation efficiency and release

Encapsulation efficiency showed marked difference in encapsulation of the two inhibitors (Table 1). Elacridar being highly lipophilic was encapsulated around 90% in all formulations irrespective of the surfactant used. Verapamil, due to its cationic character showed varying encapsulation rates depending on the surfactant used and the subsequent ionic interactions

with them. Encapsulation was highest in anionic- P+S NPs (47.9 ± 0.2%) and lowest in cationic-CTAB NPs (11.8 ± 0.6%). Cumulative release of both inhibitors showed biphasic pattern of fast initial release within the first 12hr followed by a further slow and continuous release; a characteristic of PLGA-based NPs (Figure 1).

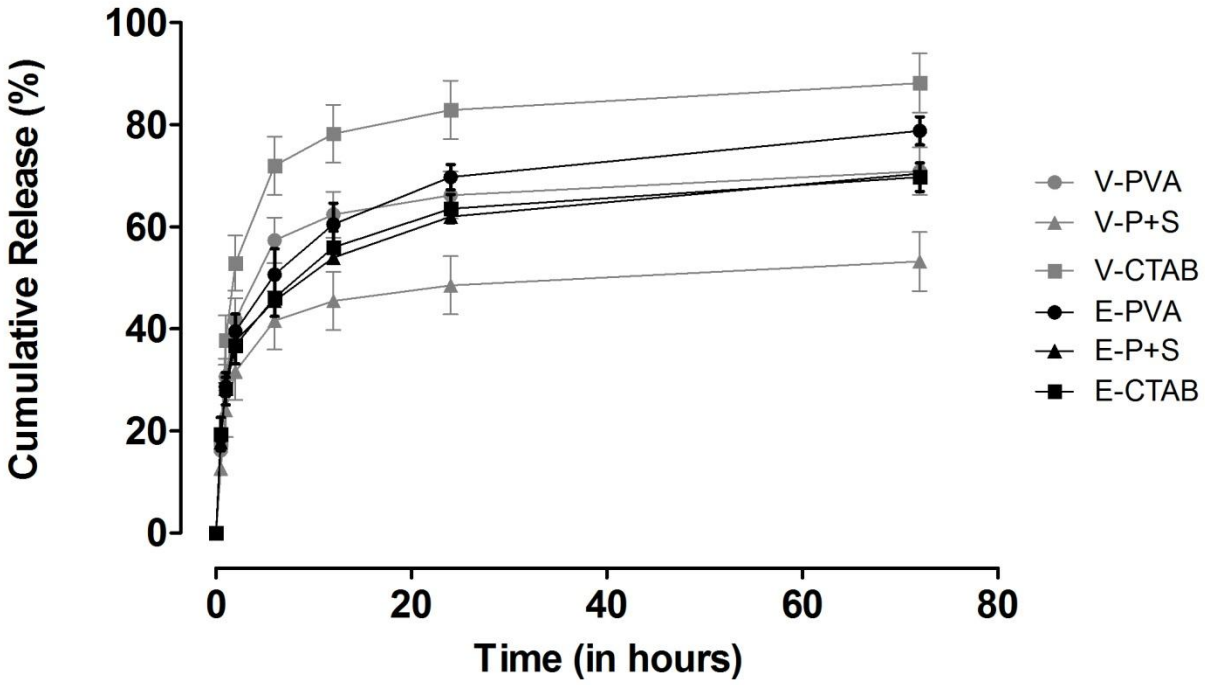

Figure 1. Cumulative release of verapamil- and elacridar-loaded nanoparticle formulations in PBS at physiological pH (7.4) plotted against time. (Mean ± SD; n = 3)

4.4.3. Confocal Microscopy

Confocal images of A2780 resistant were taken following treatment with PVA, P+S and CTAB nile red NPs (Figure 2). Interaction between all NPs and cells was higher in sensitive cells than the resistant ones as expected. Microscopic images showed strong presence of CTAB NPs exhibiting a strong interaction between NP and cell membrane after 4hr NP treatment which was followed by P+S NPs followed by PVA NPs to some extent.

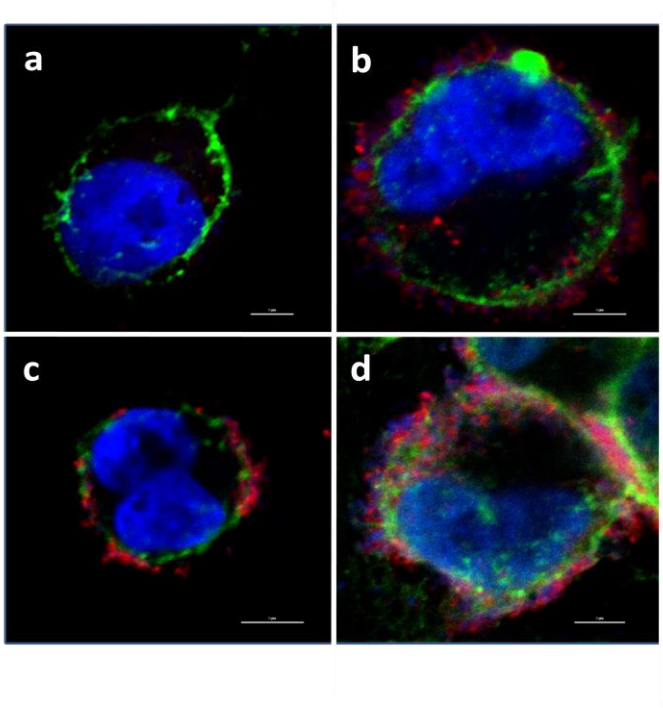

Figure 2. Confocal images of A2780Adr cell lines. The cells were incubated for 4hr with three different nile red NP formulations (red channel). **a.** control untreated; **b.** PVA NP; **c**. P+S NP; **d.** CTAB NP. The cell membrane was stained with wheat germ agglutinin (green) and the nucleus with DAPI (blue). Scale bar represents 5µm.

4.4.4. Calcein AM accumulation assay

The ability of inhibitor and their corresponding NP's to modulate P-gp function was evaluated using the calcein AM assay in P-gp over-expressing A2780Adr cells. The results depicted in Figure 3 represent rate of accumulation of calcein (a fluorescent P-gp substrate) within cells in 4.5hr span. Except blank particles of PVA and P+S, all treatment groups led to significant rate of accumulation as compared to untreated, calcein AM administered resistant cells (A2780Adr), which could be attributed to inhibitor-induced chemosensitization (except blank NPs). Owing to the higher adherence of CTAB NPs to cell membrane, all cationic formulations (blank, verapamil and elacridar) showed marked increase in calcein accumulation. As V-CTAB NPs exhibited low encapsulation and higher release rate, in order to avoid enhanced cell-adhering and uptake effects of CTAB NPs, V-CTAB NPs were used at one-fourth equivalent of VRP concentration (2.5µl/mL NP by volume) in all subsequent *in vitro* experiments. Although used at 10-fold lower concentration than VRP, elacridar and its respective formulations gave higher rates of calcein

accumulation demonstrating higher potency of the third-generation inhibitor. A combination of elacridar and CTAB NPs led to near complete sensitization of drug resistant cells at par with parental cell line (A2780).

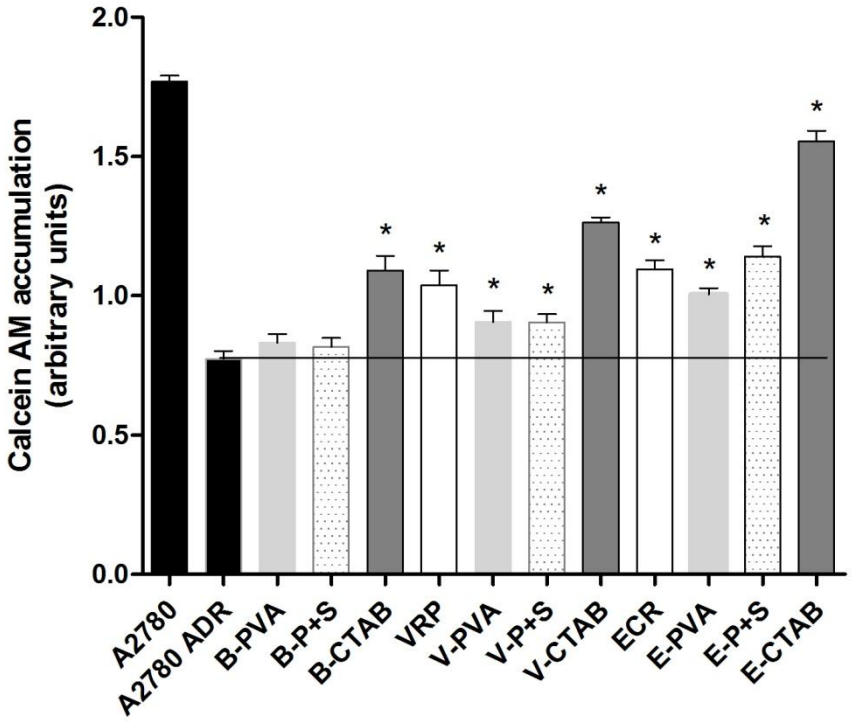

Figure 3. Calcein AM accumulation following treatment with different formulations for 4hr in KHB buffer. *$p < 0.05$ compared to untreated adriamycin resistant A2780 cells, One-way ANOVA followed by Dunnett's post test. (Mean± SD; n=3)

4.4.5. Rhodamine-123 uptake assay

Rhodamine-123, another P-gp substrate and fluorescent dye was used to study the effect of P-gp inhibition and the results were similar to those obtained with calcein. Rhodamine-123 uptake enhanced in all treatment groups as compared to the drug resistant cell lines (Figure 4). Accumulation was significantly high in both inhibitor-loaded CTAB NPs but not as significant in inhibitor solution, PVA NP and P+S NP treated cells. Elacridar-loaded CTAB NPs showed rhodamine-123 accumulation in resistant cells equal to A2780 sensitive cell lines demonstrating complete MDR reversal within 6hr of treatment.

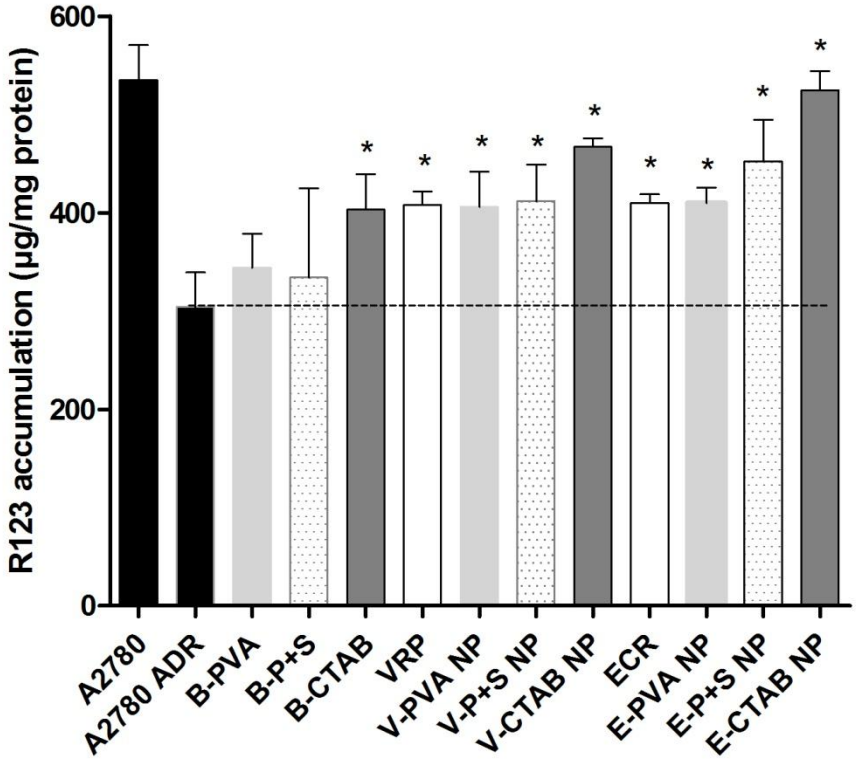

Figure 4. Rhodamine-123 accumulation assay following treatment with different formulations for 6hr in serum-free media; (Mean ± SD; n=9). ; *p < 0.05 compared to untreated adriamycin resistant A2780 cells, One-way ANOVA followed by Dunnett's post test.

4.4.6. Cytotoxicity with anti-cancer drugs

To determine the MDR reversal ability of inhibitors and their nanoparticles, the MTT assay was performed using P-gp substrate doxorubicin. Sensitive and resistant cells were incubated for 72 hr in presence of different concentrations of doxorubicin and a fixed concentration of the inhibitors or their NPs. Exposure to inhibitors and their NP formulations resulted in sensitization of resistant cells. The reversal efficiencies is defined by the following:

$$\textbf{Reversal Efficacy} = \frac{\text{Mean } GI_{50} \text{ of free anti} - \text{cancer drug (resistant cells)}}{\text{Mean } GI_{50} \text{ of treatment groups (resistant cells)}}$$

In general, as compared to doxorubicin treated drug resistant cells lines, blank-, verapamil- and elacridar-CTAB-NPs showed 3-, 20- and 193-fold lower IC_{50} values (reversal efficacies). This was followed by blank-, verapamil- and elacridar-P+S-NPs exhibiting 2-, 15- and 36- fold lower IC_{50}

values. Resistance ratio with respect to the parental cell lines showed highly significant results in both VRP and elacridar treatment groups and complete reversal (Figure 5). Unlike treatment with inhibitor solution, sustained release of inhibitors from NP core over the period of treatment can play crucial role by sensitizing the drug resistant cells towards cytotoxic drug and reverting the condition. Amongst blank NPs, CTAB particles exhibited maximum P-gp inhibition in line with previous accumulation assays followed by P+S. The ability of blank particles to inhibit P-gp transporter could be reasoned out due to their surfactant-cell membrane interactions property which has been demonstrated previously (Chapter 2 reference). All elacridar-based NPs demonstrated a complete reversal showing highest efficacy when incorporated in CTAB NPs.

	IC_{50} (nM Doxorubicin)	Reversal efficiency
A2780	5.8 ± 1.5^{a}	
A2780Adr	212.8 ± 44.4^{f}	
B-PVA NP	202.3 ± 34.1	1.2 ± 0.1
B-P+S NP	139.0 ± 51.4^{a}	1.8 ± 0.3
B-CTAB NP	94.6 ± 21.5^{a}	2.6 ± 0.0
VRP-HCl solution	52.8 ± 13.8^{a}	4.6 ± 0.2
V-PVA NP	$47.5 \pm 3.2^{a,d}$	5.1 ± 0.8^{d}
V-P+S NP	$16.4 \pm 3.2^{a,b,e}$	$14.7 \pm 0.4^{b,e}$
V-CTAB NP	$13.0 \pm 6.1^{a,b,f}$	$20.5 \pm 6.3^{b,f}$
ECR solution	34.9 ± 4.1^{a}	6.9 ± 0.7
E-PVA NP	$6.8 \pm 1.0^{a,c,d}$	$34.5 \pm 2.5^{c,d}$
E-P+S NP	$6.8 \pm 1.1^{a,c,e}$	$35.6 \pm 2.2^{c,e}$
E-CTAB NP	$1.2 \pm 0.2^{a,c,f}$	$193.3 \pm 6.5^{c,f}$

Table 2. IC_{50} values of NP formulations following MTT assay in cells for 72h. Reversal efficacy was calculated from rations between the mean IC_{50} values of free doxorubicin and that of other various formulations in A2780Adr cell lines. Data are shown as Mean ± S.D. (n=3) [a]$p< 0.05$ compared with doxorubicin treated resistant cell line. [b]$p< 0.05$ compared with verapamil hydrochloride solution. [c] $p< 0.05$ compared with elacridar solution. [d] $p< 0.05$ compared with B-PVA NP. [e] $p< 0.05$ compared with B-P+S NP. [f] $p< 0.05$ compared with B-CTAB NP.

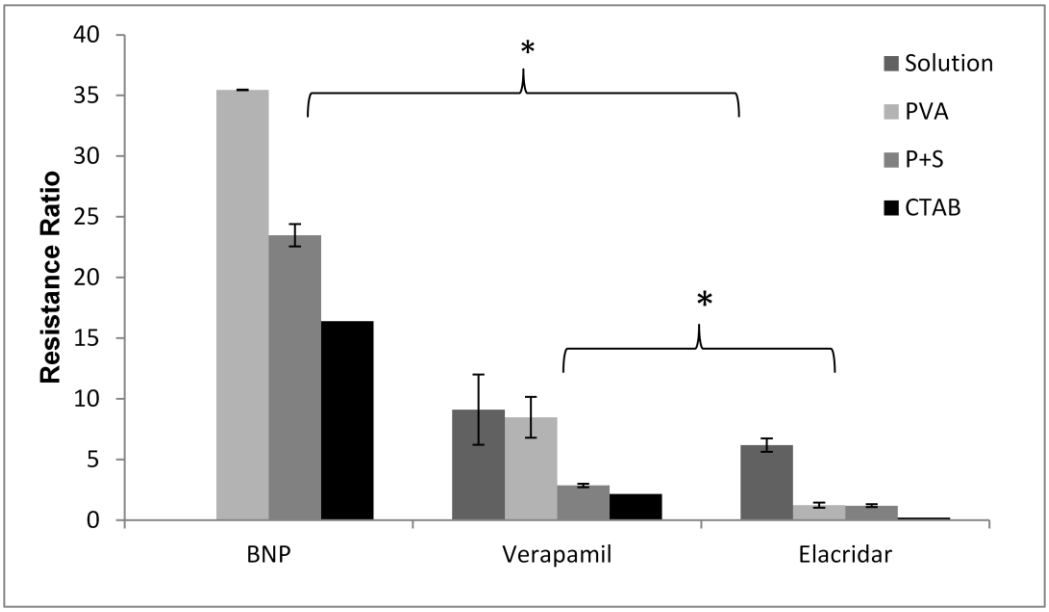

Figure 5: Resistance ratio denotes the quotient of IC_{50} value of the inhibitor/NP treated resistant cell line to that of the doxorubicin treated parental cell line. *p< 0.001 two-way ANOVA.

4.4.7. Mechanism of endocytosis in resistant and sensitive cells

In order to understand the mode of internalization of NPs in drug resistant and drug sensitive cell line, well characterized inhibitors of known endocytic pathways were used. Nanoparticles did not show much difference on the basis of surface charge in drug resistant cells (Figure 6). Clathrin-dependent uptake can be ruled out as no significant differences were observed with chloropromazine treatment. MβCD showed 70-80% inhibition of nile-red particles uptake in A2780Adr cells implying a caveolae-dependent uptake. Interestingly, in drug sensitive counterpart A2780 cells, MβCD inhibited endocytosis by only 50-60% compared to control. This corroborates with previous reports, where NP sized more than 200nm were shown to be uptaken via caveolae-dependent mechanism[11,12]. Nystatin, which seqesters cholesterol, however did not affect the uptake process in either cell lines. Dimethyl amiloride and Cytochalasin D, both of which affect macropinocytosis process did not appear to influence internalization of NP and this was expected as macropinocytosis is usually done by professional antigen presenting cells of the immune system. In drug resistant cells- approximately 40% and in drug sensitive cells- 50% uptake seems to be due to passive diffusion (4°C treatment). This

might be due to the size distribution of particles which in turn affects dose limitations to the target cells. Another notorious factor can be the adsorbed NP on cell surface which are not internalized and are difficult to remove even after repeated washing steps. Heavy concentration of CTAB NPs was also observed by confocal microscopy following 2hr incubation with nile red NPs (Figure2d). Higher accumulations of adsorbed NP on membrane surface has also been reported by Vranic et al[9] as well which affect the accurate analysis of internalization pathway. CTAB NPs (187.4± 14nm) which are on the borderline of 200nm size (< 200nm is the upper limit for clathrin-dependent endocytosis) can well be uptaken by clathrin coated vesicles (chlorpromazine treatment) but their internalization mechanism appears to differ in the two cell lines. In resistant cells, due to densely packed membrane, and intrinsic efflux property, NP internalization might be difficult. On the contrary, in sensitive cell line, a significant 23% uptake seems to be accomplished via clathrin-coated vesicles together with caveolae-dependent endocytosis (approx. 45%).

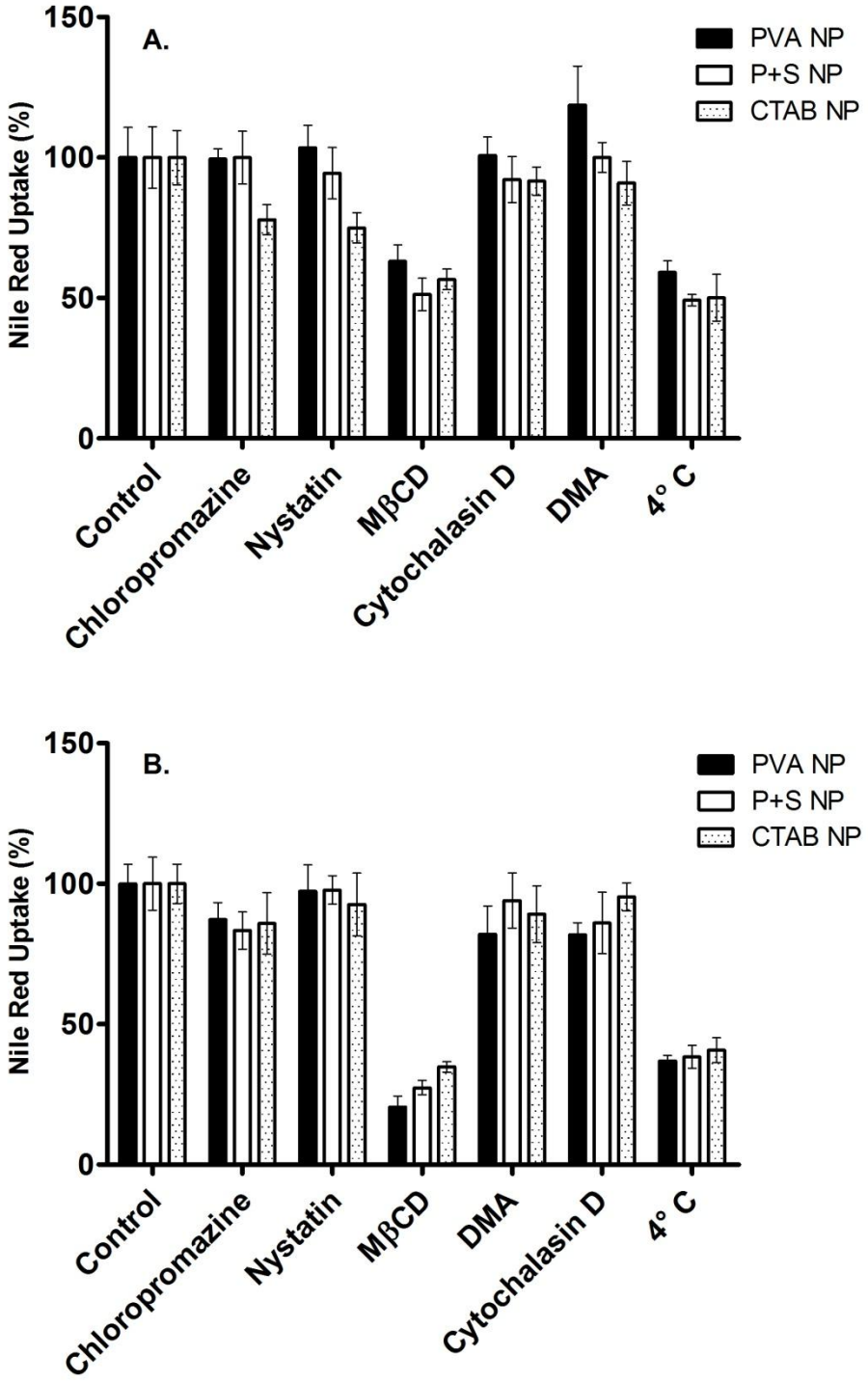

Figure 6: Nile red uptake (%) following incubation of A) A2780 and B) A2780Adr cell lines with inhibitors of endocytosis pathway and nile red NP. Active uptake was blocked by incubation at 4°C. Control group were incubated with nile red without inhibitor treatment.

4.5. Discussion

Purpose of this work was to investigate the ability of P-gp inhibitor-loaded surfactant-based nanoparticles to sensitize drug resistant cells towards co-administered P-gp substrates. First and third generation inhibitors-verapamil hydrochloride and elacridar respectively were encapsulated in non-ionic, anionic and cationic NPs. Third generation inhibitor- elacridar is more potent and the hydrophobicity can yield high entrapment efficiency. In contrast, entrapment of hydrophilic- verapamil poses challenge due to the hydrophobic nature of PLGA[13] and leaching out into aqueous phase. This reflected in both encapsulation efficiencies (Table 1) and release profile (Figure 1) of verapamil loaded NPs.

PVA-SDS NPs exhibited comparatively higher entrapment but low release of VRP in contrast to V-CTAB NPs which could be explained due to the electrostatic interactions between drug molecules and surfactant. On the other hand, elacridar showed a high rate of encapsulation in all formulations due to their highly lipophilic character. Over 50% incorporated drug was released from all formulations in first 6 hr, except V-P+S NPs (41.6 ± 5.7%) wherein a strong ionic interactions between cationic drug and anionic surfactant (SDS) could be responsible for a slower release. In contrast, rate of release of VRP was highest in CTAB particles due to repulsion between cationic surfactant CTAB and drug molecules.

In order to visualize differential NP-cell membrane interactions, nile red-loaded, surfactant-based NPs were prepared and used to treat drug resistant cells. The interaction between NPs and cells was charge-dependent. Highest adherence was observed with cationic- CTAB particles which could be due to the negatively charged cell membrane. This was followed by anionic-PVA+SDS particles and to a lesser extent PVA NPs. To assess quantitative advantage of all formulations various in vitro assays were performed with P-gp substrates.

Elacridar (0.25 µg/mL) was used at 10-fold lower concentration than the first generation inhibitor verapamil (2.5 µg/mL) in all assays. Equivalent concentrations of respective NPs were used for treatment in all *in vitro* assays except V-CTAB as aforementioned in section 3.4. Both calcein and rhodamine-123 are P-gp substrates and an increased accumulation marks P-gp

inhibition in resistant cell lines. A correlation with the inhibitor-release profile suggests almost 50% release from almost all inhibitor-loaded formulations at 6hrs; at which time frame, it was obvious that a complete sensitization is difficult to achieve as mentioned in previous work[14]. Treatment with different formulations in both assays yielded similar result profiles with CTAB-based NPs showing highest drug reversal. However, both PVA- and PVA+SDS-NPs did not show significant difference over each other although inhibitor-loaded NPs were significantly better in sensitizing drug resistant cells compared to untreated group.

In vitro cytotoxicity results with doxorubicin (72hr treatment with NP) presented an extrapolation of in vitro accumulations assays with a clear order of efficacy as follows: CTAB NP > PVA+SDS NP > PVA NP. This was possible due to an extended period of release of inhibitors from NP and sensitization of drug resistant cells to complete reversal.

Overall the results appear to show charge and surfactant-dependence on P-gp modulatory properties of particles. Several reasons might be responsible to support our findings of higher efficacy with cationic NPs in overcoming drug resistance which have also been demonstrated recently with other cationic surfactants as well[15,16]. Drug resistant cells have altered membrane properties, such as presence of higher amounts of cholesterol and sphingomyelin[17]. Negatively charged cholesterol in drug resistant cells might be responsible for an enhanced adhesion of CTAB NPs to the cell membrane. Experiments to support this were, however, out of the scope of present study.

Uptake of particles in both drug resistant and sensitive cells was largely clathrin-independent implying involvement of either or both cholesterol-rich membrane microdomains namely caveolae and lipid rafts[18]. Methyl-β-cyclodextrin is known to knock out cholesterol out of lipid rafts, rendering the membrane more fluid[19] which in turn perturbs formation of clathrin-coated vesicles due to non-availability of cholesterol[20]. As observed in our results, with MβCD treatment (Figure 6), cholesterol-rich drug resistant cells showed almost 20% more uptake by caveolae-dependent pathway than their sensitive counterparts. However, the difference in uptake mechanisms on the basis of particle surface charge was not very prominent, which may be due to the similar size range of particles used (~170-240nm).

In a recent work, didodecyldimethylammonium bomide (DMAB) and CTAB were shown to exhibit higher biophysical interaction with the lipid extracts (measured in terms of change in surface pressure of membrane) of resistant cell than with those of non-transporter expressing endothelial cells which co-related with the higher uptake in the former[16]. Of the two surfactants, DMAB with two chains showed higher efficacy than single chained-CTAB. Cationic particles are known to induce fluidity in the lipid bilayer as well[21] which in turn has been accounted for P-gp inhibition and substrate uptake by mild detergents such as Tween® 20 or Triton™ X-100[22].

On the other hand anionic particles bind lesser and rather non-specifically to the few cationic sites present on plasma membrane causing local gelation[21]. In this work, PVA+SDS-based NPs showed relatively higher P-gp inhibition than PVA NPs. Anionic detergent SDS have been shown to enhance intracellular accumulation of epirubicin in Caco-2 cells[23] in previous studies although a specific mechanism is yet to be ascribed for the enhanced P-gp modulatory activity.

Comparable to the ongoing efforts towards achieving localized therapeutic effect of anti-cancer drug; it is of imminent importance to deliver P-gp inhibitor/s at tumor-site, due to expression of the drug resistant transporters at physiologically relevant tissues[2]. Exposure of inhibitors to non-target, non-tumor sites can lead to unfavorable pharmacokinetics of toxic compounds and have been the cause for failure of clinical trials even with inclusion of third generation inhibitors[24]. Drug delivery systems can offer 1) localized cancer therapeutics, 2) prevent undesirable pharmacokinetic interactions between P-gp modulator and P-gp substrate, and 3) sustained release of inhibitors which would ensure prolonged sensitization of drug resistant tumors towards cytotoxic drug.

As the surface of nanoparticle first to interact with cell membrane, it would be ideal to use P-gp modulatory surfactants in formulations incorporating commercial inhibitors to achieve two-stepped inhibition for complete sensitization and reversal of drug resistance. Delivery of anti-cancer drug in nanocarriers with intrinsic P-gp modulatory constituents is showing promising results[2,3]. Moreover, efforts are underway to characterize routinely used excipients which as

component of nanoformulation would be able to inhibit MDR transporters thereby overcoming MDR in cancer.

4.6. Conclusion

In this study, the effect of P-gp inhibitor-loaded, non-ionic, anionic and cationic surfactant-based nanoparticles were evaluated for their ability to sensitize drug resistant cells towards doxorubicin. Third-generation inhibitor- elacridar exhibited high encapsulation efficiency, sustained release profiles and high potency in drug accumulation and cytotoxicity assays in comparison to the first generation inhibitor-Verapamil hydrochloride based nanoparticles. Overall, cationic nanoparticles- blank or inhibitor-loaded resulted in higher sensitization of drug resistant cells towards P-gp substrates. Efficacy of both- verapamil and elacridar were enhanced when delivered via nanoparticles in longer duration treatments due to the sustained release of inhibitors and a consequent prolonged sensitization of resistant cells. As observed from this study, charge of the surfactants and their inherent membrane binding and P-gp modulatory activity can be utilized to achieve higher sensitization than achieved by delivery of inhibitor alone to improve therapeutic outcome in treating drug resistant tumors.

4.7. References

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5. Chapter 3:

Evaluation of dual P-gp-BCRP inhibitors as nanoparticle formulation

5.1. Abstract

Overcoming multidrug resistance (MDR) in cancer is a major challenge and efforts are going on to develop inhibitors against the MDR transporters P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP1) and breast cancer resistance protein (BCRP). Recently, two 4-anilinoquinazolines KCJ-160 and KCJ-199 demonstrated potential MDR reversal activity against BCRP and to a lesser extent, P-glycoprotein. These compounds were formulated as nanoparticles (KCJ-160NP and KCJ-199NP) and assessed for their multidrug resistance inhibitory activity. Particles in the size range 300-350 nm with a loading efficiency of 69% (KCJ-160NP) and 77% (KCJ-199NP) respectively were prepared. BCRP inhibition (at 1 µM equivalent) was evident as observed in Hoechst 33342 and pheophorbide A assays (in BCRP over-expressing MDCK BCRP cells) while P-gp inhibition (at 5µM equivalent) was evaluated by calcein AM and rhodamine-123 assays (in P-gp over-expressing A2780Adr cells). Cytotoxicity assay with SN-38 in MDCK BCRP cells showed complete reversal in nearly all treatment groups (solution and NP). On the other hand, cytotoxicity assay with doxorubicin in A2780Adr cells caused complete reversal in NP treated group more than observed with free solution. The results demonstrate promising inhibitory activity of both selected compounds and their nanoparticle formulations against BCRP and P-gp establishing them as dual-inhibitors. It is imperative to investigate both inhibitors in animal models of multidrug resistance owing to the presence of multiple efflux transporters in several cancer models.

5.2. Introduction

A major obstacle in cancer therapy is the development of multidrug resistance (MDR) in cancer cells. Over-expression of transporter proteins belonging to the ATP-binding cassette superfamily (ABC) on the cellular membrane is one of the leading causes for MDR[1]. ABC transporters utilize energy from ATP hydrolysis for drug efflux which reduces intracellular drug concentration to sub-therapeutic levels. At a molecular level it is generally assumed that the transporter binds the drug from the inner membrane while it diffuses into the cell, thus preventing drug entrance. Of more than 100 ABC transporters identified from prokaryotes to humans- P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) are the most characterized phenotypes that confer drug resistance. P-glycoprotein (encoded by the gene ABCB1) was the first ABC-transporter to be identified[2] followed by MRP1 (encoded by the gene ABCC1) and later BCRP (encoded by the gene ABCG2)[3]. The term BCRP is quite misleading as these transporters are not just confined to either cancer cells or breast cells. Apart from organs where both P-gp and BCRP are expressed (such as brain, intestine, liver, kidney), BCRP is present and is responsible for protective functions in placenta and stem cells[4].

With the discovery of BCRP in brain endothelial cells, it became established that P-gp is not the only efflux transporter engaged in drug efflux across the blood-brain barrier (BBB)[5]. The third-generation and a broad spectrum MDR inhibitor elacridar, as well as certain tyrosine kinase inhibitors (gefitinib and imatinib) are some of the recently reported dual inhibitors of P-gp and BCRP[6]. However, they are not very specific towards either of these ABC transporters, which in turn pose risks for physiological functioning of normal cells.

Several efforts have been undertaken to develop new potent and selective inhibitors of BCRP since its discovery. Recently, Juvale *et al*[7,8], reported several 4-anilinoquinazolines as potent inhibitors. Some of the evaluated compounds showed even higher potencies than Ko143, which is the most potent selective BCRP inhibitor known so far. In the current study, we selected two newly identified quinazoline compounds (KCJ-160 and KCJ-199) having dual activity, showing high inhibition of BCRP and to a lesser extent of P-gp. Compounds KCJ-160 inhibited BCRP with

IC_{50} of 0.19 µM and 0.23 µM in Hoechst 33342 and pheophorbide A accumulation assays respectively (Table 1). KCJ-199 also showed good BCRP inhibition with IC_{50} of 0.42 µM and 0.61 µM in Hoechst 33342 and pheophorbide A assays. Compound KCJ-160 was even potent than the known BCRP inhibitor Ko143 which produced an IC_{50} of 0.25 µM in Hoechst 33342 assay. Both KCJ-160 and KCJ-199 were also able to inhibit P-gp at lower micro-molar range, having IC_{50} of 5.85 µM and 3.49 µM respectively in calcein-AM assay. Chemical structures and inhibitory potencies (IC_{50} values) of KCJ-160 and KCJ-199 obtained in different functional assays obtained in earlier studies are given in Table 1.

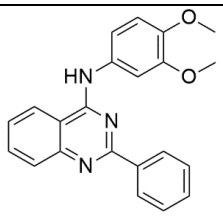
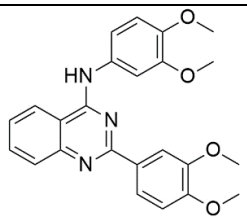
Compound	Structure	BCRP inhibition IC_{50} (µM)		P-gp Inhibition IC_{50} (µM)	clogP
		Hoechst 33342 assay	pheophorbide A assay	calcein AM assay	
KCJ-160		0.19 ± 0.01	0.23± 0.03	5.85 ± 0.79	5.529
KCJ-199		0.42 ± 0.05	0.61 ± 0.04	3.49 ± 0.23	5.282

Table 1: Structures and cLog P values of compounds KCJ-160 and KCJ-199[7,8]

Simultaneous delivery of MDR inhibitor and anti-cancer drug leads to overcoming of MDR but is hampered by potential pharmacokinetic interaction between the two administered entities. Of major limitations, is the undesirable effect on non-target normal cells where transporters play a major physiological role in effluxing xenobiotics and endogenous toxins. Hence, it is of importance to address two key issues: targeted delivery of inhibitors to tumor cells to achieve higher sensitization of the latter and to overcome unwanted pharmacokinetic interactions.

Following treatment, small molecules of anti-cancer drugs get rapidly cleared from the tissues such that their therapeutic concentrations cannot be maintained for longer durations. Also, selectivity issues in turn lead to undesired side effects of these drugs on healthy tissues. Combination therapies via drug delivery systems (such as nanoparticle based) offer multiple advantages over conventional chemotherapy[9]. On one hand they can protect the encapsulated drug/ MDR modulator from degradation *in vivo* and on the other hand they can address selectivity issues by localizing them to tumor mass. This subsequently leads to higher build up of intracellular chemotherapeutic molecule for a longer duration that can effectively kill cancer cells. Besides, MDR inhibitor loaded NP's lead to avoidance of overlapping toxicities of the co-delivered anti-cancer drug and inherent toxicity exhibited by inhibitors (if any). Over the past few years NPs have been intensively investigated as means to bypass MDR-mediated drug efflux[10]. But as cancer cells have outsmarted and evolved to express multiple efflux and survival proteins, so has the effort to develop multi-drug inhibitors that can inhibit multiple pathways/proteins.

In this study, we developed poly-lactic-co-glycolic-acid (PLGA)-based polymeric nanoparticles that have been extensively evaluated as delivery platform for a variety of drugs, peptides and genes[11]. Current literature is replete with studies involving PLGA-based nanoparticles due to their hydrophobic nature and excellent biodegradable and biocompatible properties. As the MDR inhibitors in this work (KCJ-160 and KCJ-199) are lipophilic molecules, we conceived that a high encapsulation efficiency and sustained release could be obtained using PLGA-based nanocarriers.

On the basis of high inhibitory action of the two compounds, KCJ-160 and KCJ-199 against BCRP, these compounds were evaluated as nanoparticle formulation. In addition to BCRP inhibition at very low concentrations (< 1 μM), they have demonstrated additional inhibitory activity against P-glycoprotein. In this work, we have shown enhanced cytotoxicity when these inhibitors were formulated as nanoparticles and administered with the anti-tumor drugs SN-38 and doxorubicin, which are BCRP and P-gp substrates respectively[12,13].

5.3. Materials and Methods

5.3.1. Materials

KCJ-160 and KCJ-199 were synthesized in-house with a purity of >98% (Table 1). Poly-lactide-co-glycolide (PLGA) RG 502 H was obtained from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA, Mowiol® 4-88) was a gift from Kuraray (Frankfurt, Germany) and Verapamil hydrochloride from Fagron (Barsbüttel, Germany). Hoechst 33342, calcein AM, doxorubicin hydrochloride and rhodamine-123 were purchased from Sigma-Aldrich (Steinheim, Germany). SN-38 and Ko143 were purchased from TCI Europe N.V. (Eschborn, Germany) and Tocris Bioscience (Bristol, United Kingdom) respectively. All other chemicals were of analytical grade.

5.3.2. Cell lines

Wild type MDCK and BCRP expressing MDCK cells were a kind gift from Dr. A. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). MDCK BCRP cells were generated by transfection of the canine kidney epithelial cell line MDCKII with the human wild-type cDNA C-terminally linked to the cDNA of the green fluorescent protein (GFP). These cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10 % fetal bovine serum (FBS), 50 µg/mL streptomycin, 50 U/mL penicillin G and 2 mM L-glutamine. The human ovarian carcinoma cell line A2780 and the corresponding P-gp overexpressing doxorubicin resistant A2780Adr cell line were purchased from ECACC (Nos. 93112519 and 93112520). The cell lines were grown in RPMI-1640 medium supplemented with 10% FBS, 50 µL/mL streptomycin, 50 U/mL penicillin G, and 2 mM L-glutamine. All cell lines were cultivated in a 37 °C incubator with 5% CO_2/ 95% humidified air. Subculturing was performed after confluence of 80-90%, using 0.05 % trypsin and 0.02 % EDTA. Cell counting for different assays was performed using a CASY1 model TT cell counter with 150 µm capillary (Schaerfe System GmbH, Reutlingen, Germany).

5.3.3. Preparation and characterization of nanoparticles

NP's were prepared by dissolving 20 mg PLGA and 1 mg each of KCJ-160 and KCJ-199 in 400 µL dichloromethane as internal phase. This was poured in 2 mL of 2% (w/v) PVA solution (aqueous phase) and ultrasonicated (50% duty cycle for 2 min). The organic phase of internal phase was evaporated by stirring for 3hr. Blank nanoparticles were prepared analogously without the addition of either inhibitor. Nanoparticles were analyzed for their size distribution, polydispersity index (PDI) and zeta potential using a ZetaPlus (Brookhaven Instruments Corporation). Each sample was analyzed in triplicate.

5.3.4. Encapsulation efficiency and inhibitor release

The amount of inhibitor entrapped within the NPs was detected by RP-HPLC using a column Eurospher II 100-5 C18H (50 x 4 mm) connected with a pre-column Vertex-Plus Eurospher II 100-5 C18H (5 x 4 mm) obtained from Knauer, Germany. Eluent (methanol:water (70:30)) flow rate was maintained at 1.0 mL/min and samples were detected at 254 nm.

In order to assess the drug release, 2 mL of each preparation was taken in triplicate and kept at 37° C in a shaking water bath. Phosphate buffered saline (pH 7.4) was chosen as release buffer. 0.1 % tween 80 was added to maintain sink conditions. At predefined time points 0.5 mL of release medium was withdrawn and replaced by 0.5 mL of fresh buffer for continuous release. 0.5 mL of methanol was added to the withdrawn medium, vortexed and centrifuged to extract the released inhibitor. The supernatant was quantified for the respective MDR inhibitor by HPLC as mentioned before.

5.3.5. BCRP inhibition assays

Both Hoechst 33342 and pheophorbide A are BCRP substrates and subsequently all assays were performed on sensitive MDCK and BCRP expressing MDCK cell lines. Ko143 was used as positive control and all treatments were given at a concentration of 1µM of inhibitors and their equivalent NP dose.

5.3.5.1. Hoechst 33342 accumulation assay

To investigate the effect of encapsulated target compounds on BCRP, a Hoechst 33342 accumulation assay was performed as described earlier with small modifications[14]. Following trypsization and centrifugation of MDCK and MDCK BCRP cells, the cell density was determined using a Casy I Model TT cell counter device. Followed by repeated centrifugation cells were washed three times with Krebs-Hepes buffer (KHB) and seeded into black 96-well plates (Greiner, Frickenhausen, Germany) at a density of approximately 20,000 cells per well in a volume of 80 µL. 100 µL of 1 µM inhibitors KCJ-160 and KCJ-199 and their NP equivalent were added and incubated for 30mins. After this pre-incubation period, 20 µL of Hoechst 33342 solution was added to each well yielding a final Hoechst 33342 concentration of 1 µM. Fluorescence was measured immediately in constant intervals (60s) up to 120 min at an excitation wavelength of 355nm and an emission wavelength of 460nm using a BMG POLARstar microplate reader (BMG Labtech, Offenburg, Germany) maintained at 37 °C. For data analysis, obtained from the assay, the average of fluorescence values in the steady state (from 111 min to 120 min) was calculated for each concentration. From the obtained plateau fluorescence values, concentration response curves were generated by nonlinear regression using the four-parameter logistic equation with variable Hill slope (GraphPad Prism v. 5.0, San Diego, CA, USA).

5.3.5.2. Pheophorbide A Assay

To re-evaluate the effect of encapsulated target compounds on BCRP using another BCRP substrate, a pheophorbide A assay was performed[14,15,16]. For performing the pheophorbide A assay, cells were prepared in the same manner as described for the Hoechst 33342 assay. The assay was done in a clear, U-shaped 96-well plate, with a final concentration of pheophorbide A of 0.5 µM. Following an incubation period of 2hr, cells were again re-suspended using a multichannel pipette. Fluorescence was measured using a FACSCalibur flow-cytometer. Concentration-response curves were generated by nonlinear regression using the four-parameter logistic equation using GraphPad Prism (v. 5.0, San Diego, CA, USA).

5.3.6. P-gp inhibition assays

Both calcein AM and rhodamine-123 are P-gp substrates and subsequently all assays were performed on sensitive A2780 and P-gp expressing A2780Adr cell lines to evaluate P-gp inhibitory activity of compounds and their NP's. Verapamil hydrochloride was used as positive control and all treatments were at a concentration of 5 µM of inhibitors and their equivalent NP dose.

5.3.6.1. Calcein AM accumulation assay

For determining the effect of encapsulated target compounds on P-gp a calcein AM accumulation assay was performed as described earlier with small modifications[14,17]. Cells were prepared in the same manner as described for the Hoechst 33342 assay. After a 30 min pre-incubation period, 20 µL of a 2.5 µM calcein AM solution was added to each well. The fluorescence was measured immediately in constant time intervals (60s) up to 360 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm with a BMG POLARstar microplate reader maintained at 37°C.

5.3.6.2. Rhodamine-123 uptake assay

To re-evaluate the effect the inhibitors on P-gp, a rhodamine-123 accumulation assay was performed as it is a known P-gp substrate. 10,000 cells each of A2780Adr and A2780 were seeded in 96 well plates and incubated overnight for adherence. On the next day, inhibitor and equivalent NP treatment were given at 5µM together with rhodamine-123 solution yielding a final concentration of 0.3 µM. The total volume per well was kept at 100µL. Following incubation for 6hr, media was removed, and wells washed once with PBS. 100µL Triton-X 100 (1% v/v) was added to each well and incubated for 30min to lyse cells and extract rhodamine-123. The fluorescence was measured (ex = 505 nm, em = 540 nm) with a fluorescence spectrophotometer (Victor3, Perkin Elmer) and normalized for cellular protein levels as determined by bicinchoninic acid (BCA) assay.

5.3.7. Confocal Laser Scanning Microscopy

Cells were grown over coverslips in 24-well plate and incubated with doxorubicin (0.5µg/mL), both inhibitors and their respective formulations for 6hr. Free doxoorubicin was removed by several washing steps followed by incubation with wheat germ agglutinin-FITC at 4°C for 30min. Fixation was performed with paraformaldehyde (4%) followed by nuclear staining with DAPI (300nM; 5min). Slides were prepared with glycerol/PBS and imaged with a Biorad Laser Scanning Confocal Imaging System

5.3.8. Cytotoxicity with doxorubicin

To check the effectiveness of encapsulated inhibitors to reverse the drug resistance in BCRP (MDCK BCRP) and P-gp (A2780Adr) over-expressing cells to SN-38 and doxorubicin respectively, MTT cytotoxicity assays were performed. The sensitive counterparts of these cells were used as controls. The assay was performed as described earlier with minor modifications[14,18]. In brief, cells were seeded overnight into 96-well tissue culture plates (Sarstedt, Newton, USA) at a density of 3000 cells (for MDCK cells) or 5000 cells (for A2780 cells) per well in a volume of 80 µL for adherence. Cells were treated with cytotoxic compounds (SN-38 or doxorubicin) at different concentrations in a volume of 10 µL. Target compounds and encapsulated compounds were added to each well at a final concentration of 1 µM (BCRP) or 5 µM (P-gp) to achieve the final volume of 100 µL. Control experiments were performed with medium containing 10% (v/v) DMSO. After an incubation period of 72hr, the MTT reagent was added (20 µL of a 5 mg/mL solution) to each well. Plates were further incubated for 1hr and after that the supernatant were removed from wells. Formazan crystals formed were solubilized by adding 100 µL DMSO per well. Viability of cells was measured by taking absorbance at 544 nm and background corrected at 710 nm (BMG POLARstar microplate reader).

5.3.9. Statistical Analysis

All the experiments were performed in triplicates and reported as mean ± S.D. Statistical comparisons were made with one-way ANOVA followed by Dunnett's post test using GraphPad

Prism software version 5.0. In all cases, $p < 0.05$ was considered to be significant at 95% confidence level.

5.4. Results

5.4.1. KCJ-160 & KCJ-199 loaded polymeric nanoparticles

The physicochemical properties of the three formulations are shown in Table 2. Blank and inhibitor (KCJ-160 and KCJ-199) loaded nanoparticles did not differ much in size ranging from 315 to 358 nm. Encapsulation efficiency as determined by HPLC was influenced by the slight difference in lipophilicity of the compounds, wherein the more lipophilic inhibitor KCJ-160 showed comparatively higher encapsulation.

	Size (nm)	PDI	Zeta potential	Encapsulation efficiency (%)
Blank NP	358 ± 8	0.04 ± 0.02	-12.8 ± 1.2	-
KCJ-160 NP	315 ± 14	0.05 ± 0.03	-11.4 ± 0.2	77.1 ± 0.5
KCJ-199 NP	327 ± 11	0.04 ± 0.01	-10.6 ± 0.3	69.0 ± 1.6

Table 2. Nanoparticle size, PDI, zeta potential and encapsulation efficiency of inhibitors (n=3, mean ± S.D.)

5.4.2. Encapsulation efficiency and release

Cumulative release of dual MDR inhibitors showed a sustained release profile with the more lipophilic inhibitor KCJ-160 showing comparatively slower release rate (Figure 1). The release was observed for over a week (one week data not shown) and samples analyzed by HPLC. About 46% and 54% of KCJ-160 and KCJ-199, respectively, were released in first 6hr and most assays were performed with incubation times ranging 2-6hr.

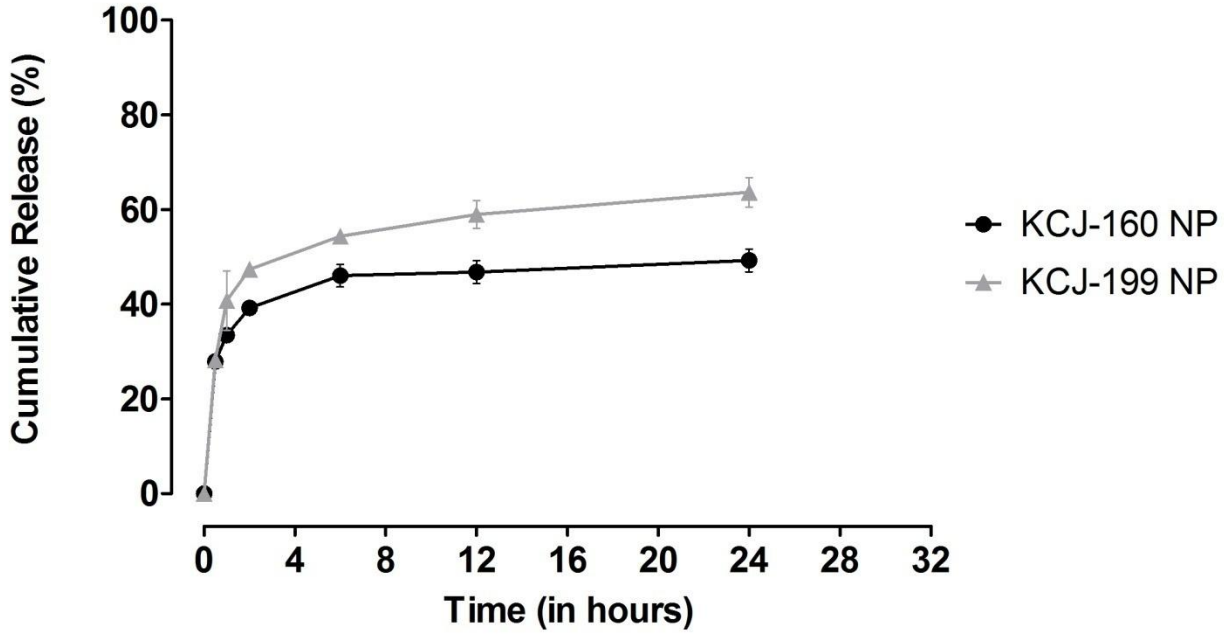


Figure 1. Cumulative release profile of KCJ-160 and KCJ-199 from NP in PBS (pH 7.4) at different time points (n=3, mean ± S.D.)

5.4.3. BCRP inhibition

On the basis of highly potent inhibitory action of the two compounds, KCJ-160 and KCJ-199 against BCRP, these compounds were evaluated as nanoparticle formulation. Two well characterized BCRP substrates, Hoechst 33342 and pheophorbide A were used as probes to assess BCRP inhibition in BCRP over-expressing MDCK cell lines. In both the assays, the potent inhibitor Ko143 was used as a positive control. Ko143 is a non-toxic analogue of fumitremorgin C (FTC) and the only known specific and potent modulator of BCRP with high affinity to the latter[16].

5.4.3.1. Hoechst 33342 assay

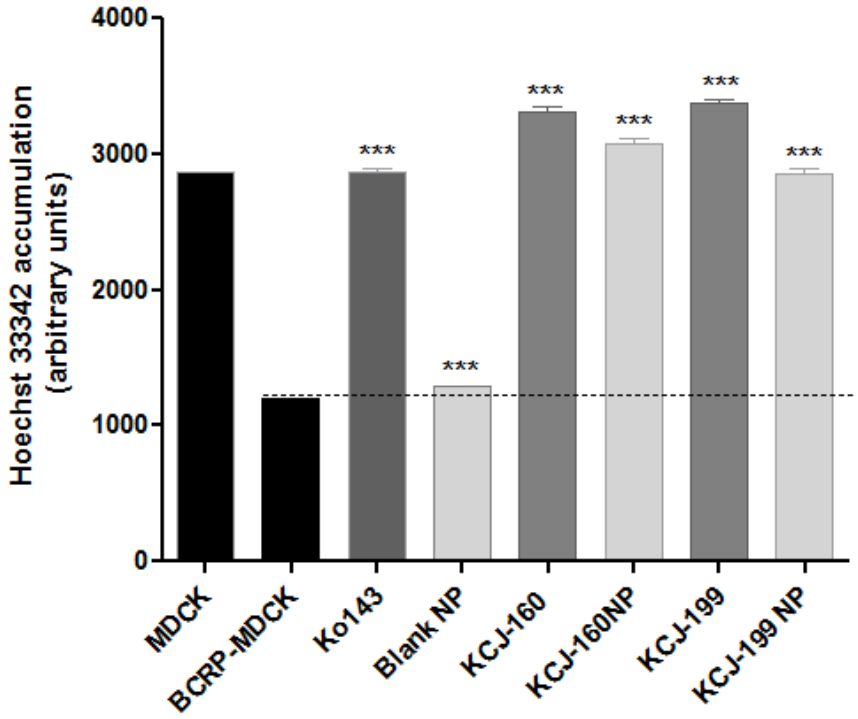
Hoechst 33342 influx across cell membrane increases when BCRP is inhibited by a modulator. This leads to an increase in fluorescence of cellular Hoechst 33342 implying efficient BCRP inhibition. We observed high accumulation of Hoechst 33342 in BCRP overexpressing cells within 2.5hr of inhibitor and equivalent NP dose treatment (Figure 2A). Both inhibitors were

potent enough at a concentration as low as 1µM resulting in complete reversal of drug resistance.

5.4.3.2. Pheophorbide A assay

Pheophorbide A, a chlorophyll-breakdown product is another known BCRP substrate. As it diffuses readily across the cell membrane, the accumulation per cell can be measured using flow cytometry. In BCRP overexpressing cells, pheophorbide A accumulation was enhanced following treatment with inhibitors (both solution and NP) (Figure 2B). These results were similar to those obtained in Hoechst 33342 accumulation assay. KCJ-160 which showed better accumulation (as both free solution and NP) as compared to KCJ-199 has been shown previously[8] to be more potent in Pheophorbide A assay (Table 1). Consequently, KCJ-199 NP did not show much potency either as the free inhibitor itself.

2A.



2B

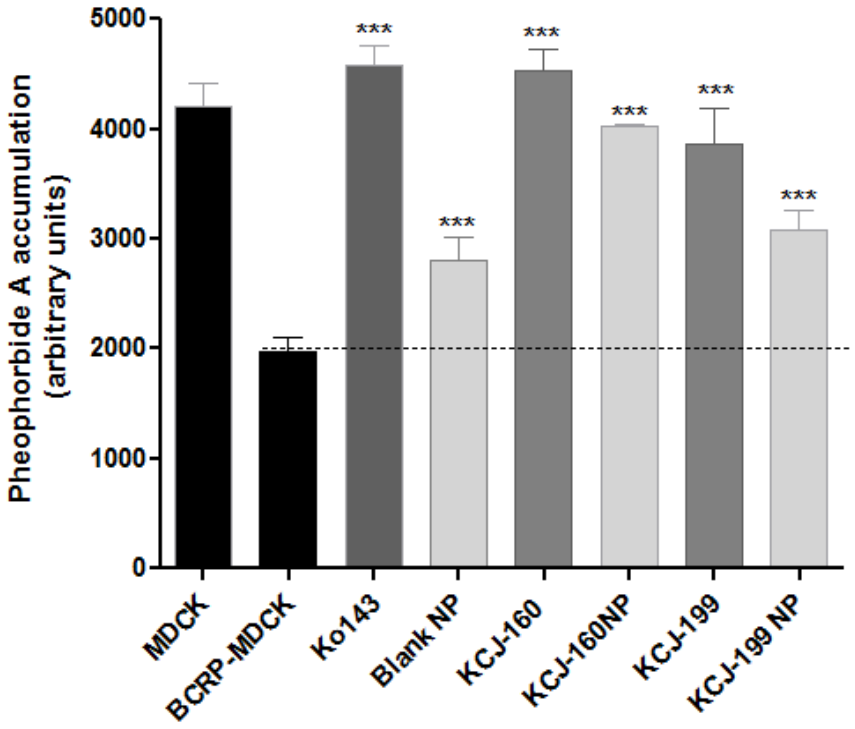


Figure 2A. Hoechst 33342 accumulation assay in BCRP expressing MDCK and sensitive cells. **2B.** Pheophorbide A accumulation assay in BCRP expressing MDCK and sensitive cells. (Mean ± S.D.; n = 6; *p < 0.001, One-way ANOVA followed by Dunnett's multiple comparison test). The dotted line represents basal Hoechst 33342 or pheophorbide A accumulation in resistant cells without any treatment.

5.4.4. P-gp inhibition assay

5.4.4.1. Calcein AM accumulation assay

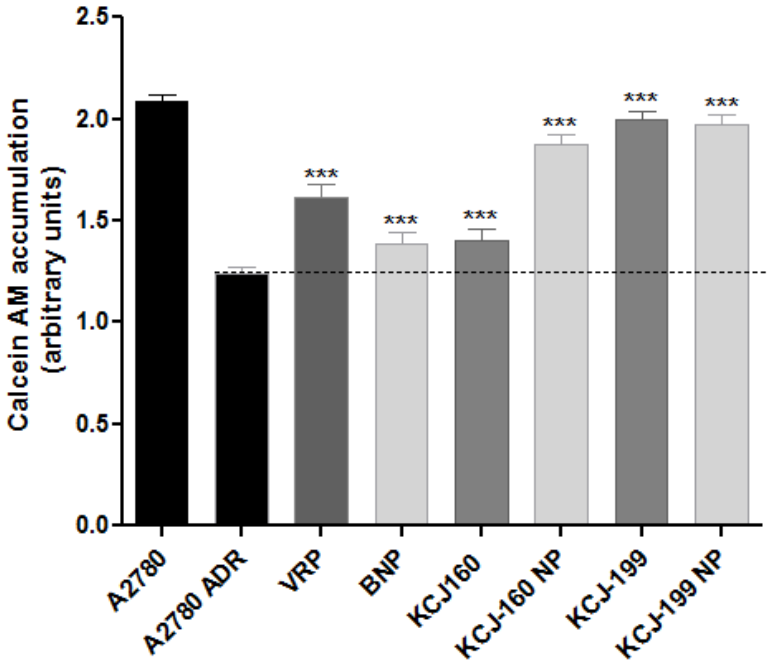
The ability of inhibitor and their corresponding NP's to modulate P-gp function was evaluated using the calcein AM assay in P-gp over-expressing A2780Adr cells (Figure 3A). Following diffusion, calcein AM gets hydrolyzed to calcein anion by esterases present in cells. Unlike calcein AM, calcein is strongly fluorescent and being hydrophilic, cannot leave cells by diffusion. In P-gp overexpressing resistant cells, the fluorescence increase caused by intracellular calcein was measured as a function of P-gp inhibition. KCJ-160 has been previously shown[8] to possess P-gp inhibitory activity to a lesser extent as compared to KCJ-199. But when encapsulated in particles, KCJ-160 shows an increased calcein accumulation. This could be possible due to

higher cellular uptake of nanoparticles as well as prolonged sensitization of cells to the inhibitor (6hr incubation in this assay). In contrast, solution and NP formulation of KCJ-199 showed high inhibitory activity on P-gp, leading to accumulation of calcein equivalent to that in sensitive cells.

5.4.4.1. Rhodamine-123 uptake assay

Rhodamine-123, a fluorescent dye which is readily effluxed by P-gp expressing cells is routinely used to study the MDR phenomenon. Rhodamine-123 uptake increased in all treatment groups including blank NP (Figure 3B). Accumulation was slightly enhanced with both KCJ-160 and KCJ-199 (solution and NP) as compared to the first generation P-gp inhibitor verapamil used as positive control. However, all treatment groups showed high P-gp inhibition leading to increased rhodamine-123 accumulation at par with the uptake in A2780 cells. Following 6hr incubation, both NP preparations showed better uptake.

3A.



3B.

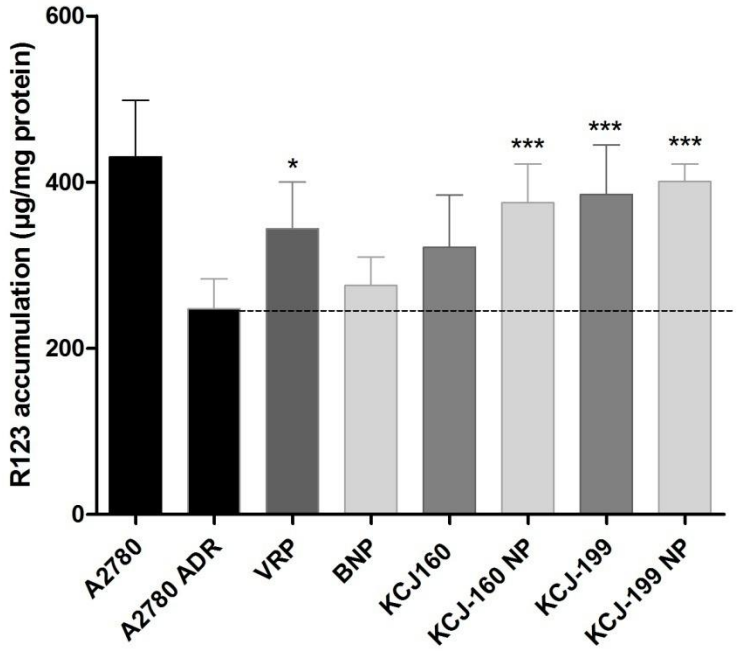


Figure 3A. Calcein accumulation assay in P-gp expressing A2780Adr and sensitive A2780 cells. **3B**. Rhodamine-123 accumulation normalized against protein levels in P-gp expressing A2780Adr and sensitive A2780 cells. (Mean ± S.D.; n = 6; *p < 0.001 compared with A2780Adr, one-way ANOVA followed by Dunnett's multiple comparison test). The dotted line represents basal calcein and rhodamine-123 accumulation in resistant cells without any treatment.

5.4.5. Confocal microcsopy

Chemosensitization of resistant and sensitive cells were performed to qualitatively analyze doxorubicin uptake (Figure 4). After 6hr treatment, drug resistant cells A2780Adr showed little doxorubicin uptake (Figure 4a.) in contrast with sensitive cell lines- A2780 (Figure 4b.). MDR reversal was observed in A2780Adr cells treated with KCJ-199 solution, KCJ-160NP and KCJ-199NP. However, the uptake was not increased significantly in KCJ-160 solution treatment group.

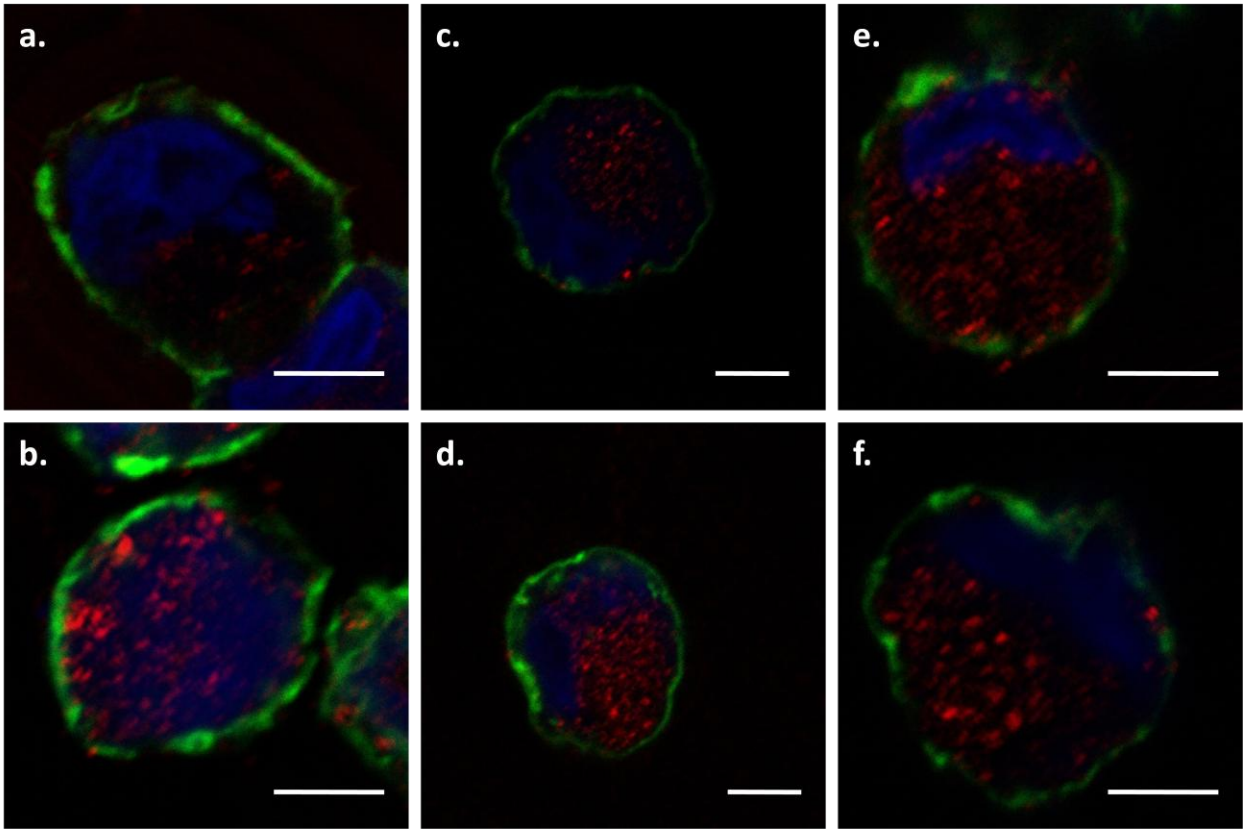


Figure 4. Confocal images of A2780Adr and A2780 cell lines. The cells were incubated for 6hr with different inhibitors and their formulations and doxorubicin (red channel). **b.** A2780- doxorubicin treated; while rest are A2780Adr images treated with free doxorubicin and **a.** A2780Adr- doxorubicin alone; **c**. KCJ-160 solution; **d.** KCJ-160 NP; **e.** KCJ-199 solution; **f.** KCJ-199 NP The cell membrane was stained with wheat germ agglutinin-FITC (green) and the nucleus with DAPI (blue). Scale bar represents 5µm.

5.4.6. Cytotoxicity with doxorubicin

To determine the MDR reversal ability of inhibitors and their nanoparticle counterparts, the MTT assay was performed using the BCRP substrate SN-38 and the P-gp substrate doxorubicin. Sensitive and resistant cells were incubated with cytotoxic compounds (SN-38 or doxorubicin) and a fixed concentration of the inhibitors or their NPs for 72hr. Exposure to dual inhibitors and their NP formulations resulted in sensitization of resistant cells. In both BCRP and P-gp expressing cells, NP's gave encouraging results with lower growth inhibition-GI_{50} values achieving complete reversal. This was largely due to sustained release of inhibitors from within nanoparticle over the period of treatment.

Marked reduction in GI_{50}-values of SN-38 in BCRP over-expressing MDCK BCRP cells were observed following treatment with KCJ-160 (solution and NP) and KCJ-199 NP (Figure 5). KCJ-199 solution exhibited slightly less efficacy as compared to KCJ-160, which was also observed in Hoechst 33342 (Figure 2A) and pheophorbide A assays (Figure 2B).

NP-based inhibition of P-gp over-expressing, A2780Adr cells gave very promising results (Figure 6). KCJ-160 NP and KCJ-199 NP were able to overcome MDR efflux with a reversal efficacy of more than 3-fold than any of the inhibitors (Verapamil, KCJ-160 & KCJ-199) administered as free solutions. Reversal efficacies of both KCJ-160 and KCJ-199 formulations were higher than that of their respective solutions (Table 4).

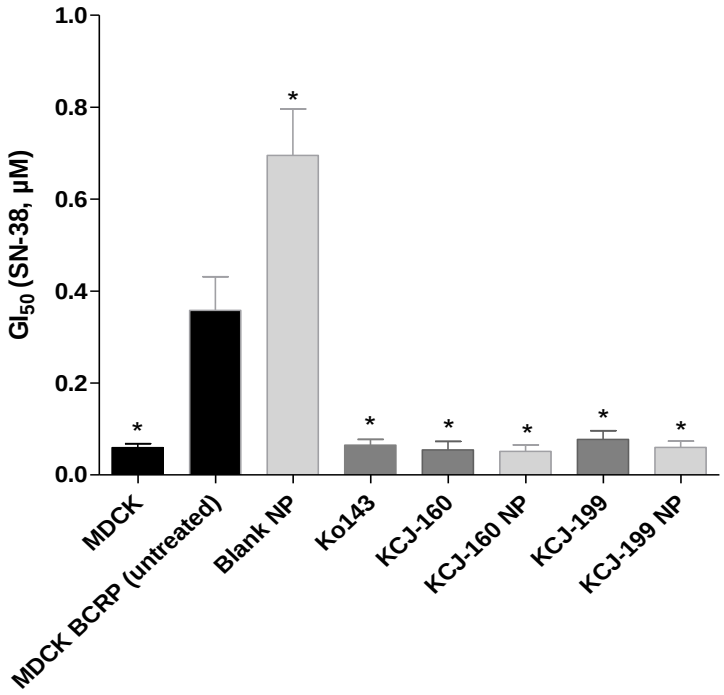


Figure 5. GI_{50} values of SN-38 in BCRP over-expressing MDCK cells and wild type (MDCK) as calculated by the MTT assay. (Mean ± S.D.; n = 3; *$p < 0.05$ compared to MDCK BCRP (untreated), one-way ANOVA followed by Dunnett's post test.)

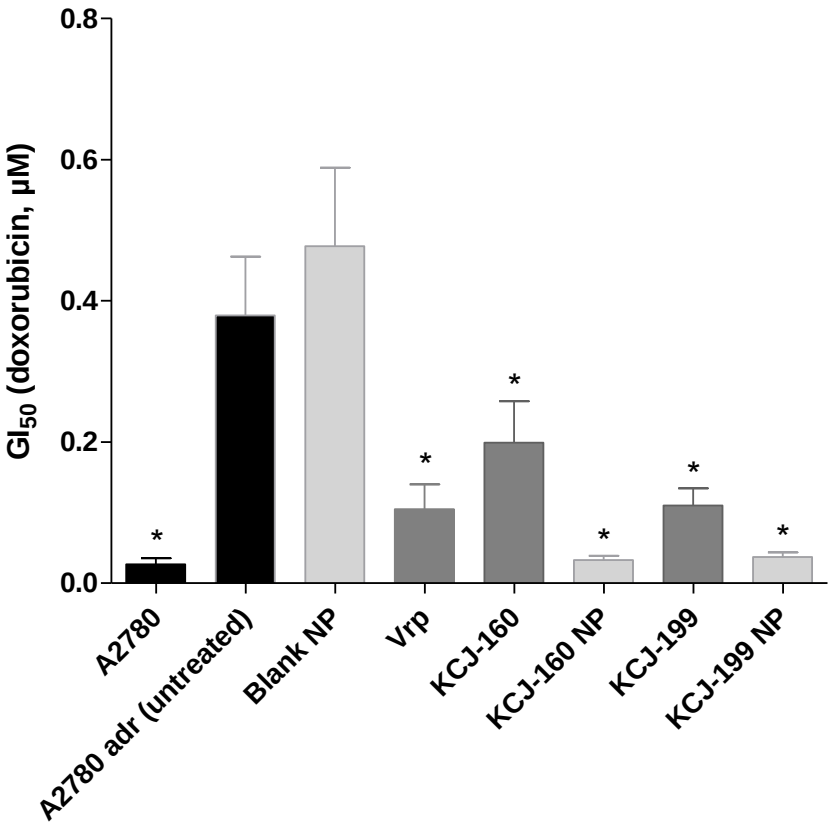


Figure 6. GI_{50} values of doxorubicin in P-gp over-expressing A2780Adr cells and wild type (A2780) as calculated by the MTT assay. (Mean ± S.D.; n = 3; *p < 0.05 compared to MDCK BCRP (untreated), one-way ANOVA followed by Dunnett's post test.)

Reversal efficacy and resistance factor were calculated from GI_{50} values with the following formulas:

$$\textbf{Reversal Efficacy} = \frac{\text{Mean } GI_{50} \text{ of free anti} - \text{cancer drug (resistant cells)}}{\text{Mean } GI_{50} \text{ of treatment groups (resistant cells)}}$$

$$\textbf{Resistance Factor} = \frac{\text{Mean } GI_{50} \text{ of treatment group (resistant cells)}}{\text{Mean } GI_{50} \text{ of free anti} - \text{cancer drug (sensitive cells)}}$$

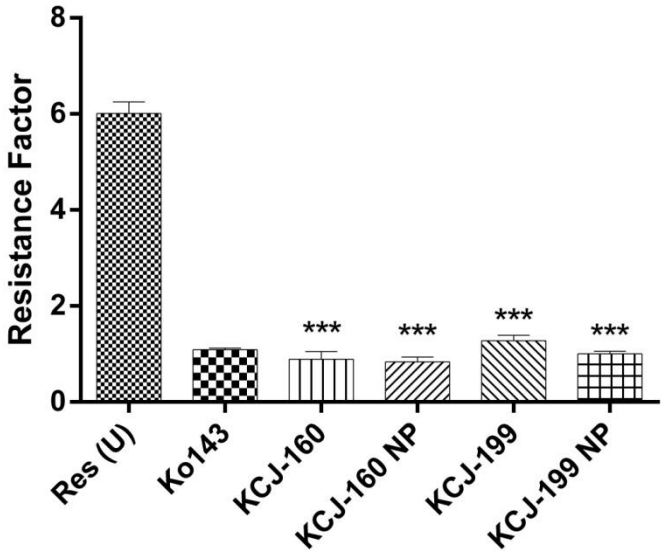


Figure 7A. Resistance factor is the quotient of GI_{50} value of treatment group in BCRP over-expressing cell line to that of the untreated sensitive cell line (MDCK). Res (U) refers to untreated resistant cell line MDCK-BCRP. (*p< 0.05 by one-way ANOVA followed by Bonferroni post t-test.)

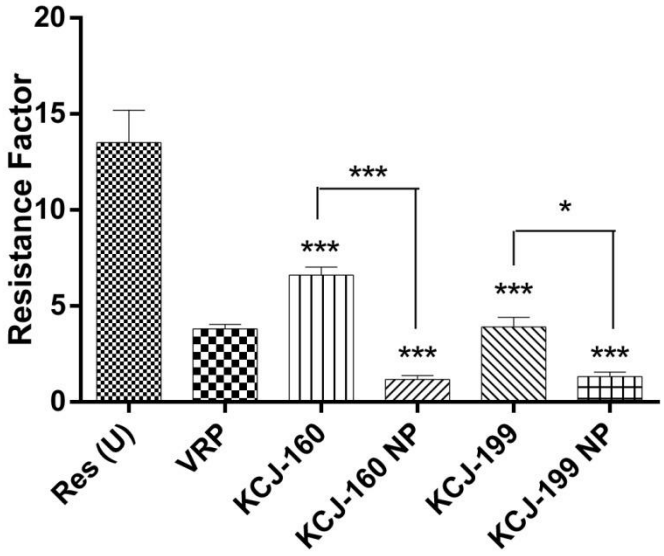


Figure 7B. Resistance factor is the quotient of GI_{50} value of treatment group in P-gp over-expressing cell line to that of the untreated sensitive cell line (A2780). Res (U) refers to untreated resistant cell line A2780Adr. (*p< 0.05 by one-way ANOVA followed by Bonferroni post t-test.)

5.5. Discussion

Two newly synthesized 4-anilinoquinazoline compounds- KCJ-160 and KCJ-199 were chosen for present work due to their ability to inhibit both MDR transporters BCRP and P-gp. PLGA-based nanoparticles of both inhibitors were developed by solvent evaporation technique. Particles in the size range 300 to 365 nm showed good polydispersity index and negative surface charge due to PLGA (Table 2). Both compounds possessing high lipophilicity had an effect on encapsulation efficiency and rate of release from nanoparticles. KCJ-160, being more lipophilic than KCJ-199 showed comparatively higher encapsulation efficiency (77.1 ± 0.5%) in nanoparticles and a slower rate of release (Figure 1) which could be explained due to increased hydrophobic interactions with the polymer PLGA. We used PLGA-based nanoparticles as hydrophobic compounds tend to release at a sustained rate via diffusion of drug from the polymer matrix, which may prolong inhibitory action and thereby higher sensitization of resistant cells towards cytostatic drugs can be achieved.

The efficiency of both inhibitors are proven[8] with a high potency against BCRP but to a lesser extent against P-gp. Two pairs of cell lines that over-express each transporter (BCRP and P-gp) were used in this study. The most characterized and quantifiable substrates were selected for each transporter. As positive controls, inhibition of each transporter was evaluated with a known inhibitor (Ko143 and verapamil).

The inhibition activity of both the inhibitors and their NP counterparts were indicated by their ability to inhibit efflux of fluorescent substrates- Hoechst 33342 and Pheophorbide A in BCRP overexpressing MDCK-BCRP cells. Increased accumulation of Hoechst 33342 (Figure 2A) and Pheophorbide A (Figure 2B) were observed with both inhibitors and at equivalent concentration of NPs. Accumulation of fluorescent substrates in the sensitive cell line- MDCK, did not change in the presence or absence of either inhibitors or nanoparticles (data not shown). Complete reversal of drug resistance could be demonstrated (equivalent to parental cell line) well within 2.5hr of assay. KCJ-199 is less potent than KCJ-160 in inhibiting BCRP against Pheophorbide A accumulation (Table 1) which is consistent with results obtained in the work of Juvale et al[8].

This could be extrapolated to explain lower Pheophorbide A accumulation with KCJ-199 NP treatment.

Accumulation of the fluorescent substrates- calcein and rhodamine-123 in P-gp over-expressing A2780Adr were evaluated as markers for inhibitory potency of both compounds and their NPs. As these inhibitors have lower P-gp inhibition ability, a prolonged incubation (6hr) at higher concentrations (5µM) of inhibitor and respective NPs were carried out to achieve maximum sensitization. In line with observations of Juvale et al[8], KCJ-160 showed lower inhibition of P-gp as compared to KCJ-199. However, it is interesting to note that although KCJ-160 does not possess high inhibitory activity against P-gp, when delivered via nanoparticle, it showed significantly high inhibition in both assays. This might be due to the intracellular delivery of KCJ-160 NPs within cells from where the inhibitor can exert a higher action against P-gp transporter molecule. KCJ-160 NP, KCJ-199 solution and NP demonstrated high inhibition potency against P-gp in terms of accumulation of calcein and rhodamine-123.

To reaffirm the results obtained with fluorescent substrates, the inhibitory potential of compounds and NPs was also evaluated by checking their effect on resistance of cells towards cytotoxic substrates of each transporter. For this purpose, anti-cancer drugs SN-38 (BCRP substrate) and doxorubicin (P-gp substrate) were used in MTT assay. As an extrapolation of Hoechst and pheophorbide A assay, complete reversal of resistance in BCRP over-expressing cells (MDCK-BCRP) was achieved, marked by GI_{50} value of SN-38 nearing that of parental cell line (MDCK). In P-gp over-expressing cell lines, inhibitor loaded nanoparticles gave much higher sensitization than the inhibitors alone. Fluorescent substrate assays with both calcein and rhodamine-123 did not show clear result in terms of complete sensitization and statistical significance amongst the groups. Only KCJ-160 showed similar results and demarcated lower inhibition potency against P-gp transporters as compared to KCJ-199, even with doxorubicin. Confocal images of drug resistant and sensitive cell lines demonstrated similar results as the accumulation assays for P-gp inhibition with very high uptake of doxorubicin in both NP treated groups (Figure 4d,f.).

Following 72h incubation with doxorubicin and both the inhibitors and their respective NPs, very encouraging results were obtained. KCJ-160 and KCJ-199 NPs decreased the resistance of P-gp cell lines to doxorubicin by over 5-fold ($p< 0.001$) and over 2-fold ($p< 0.01$) as compared to their free solution respectively (Figure 7A). Both inhibitors, which possess lesser activity against P-gp transporter demonstrated almost complete reversal of drug resistance when delivered as nanoparticles. This might be due to sustained rate of release of inhibitor from the PLGA matrix over a period of time leading to sensitization of resistant cells to near completion and eventually killing them. However, in BCRP over-expressing cells, sensitization achieved with solution and NP formulations were at par showing equal therapeutic efficiency (Figure 7B).

Although several efforts have been taken to identify selective inhibitors of BCRP as well as P-gp, broad-spectrum MDR modulators have the advantage of countering multiple resistance mechanisms that could be present in the therapy resistant tumour/malignant cells. It has been observed that clinically important substrates of ABCG2 may possess overlapping substrate specificity also for P-gp. Few of these substrates include anticancer compounds such as topotecan, gefitinib, erlotinib, flavopiridol and mitoxantrone. In such cases broad spectrum ABC transporter inhibitors could be advantageous.

This study highlights the importance of nanocarriers in delivery of MDR inhibitors. A delivery platform not only helps improve solubility issues of lipophilic compounds, when injected intravenously nanoparticles can increase inhibitor/ drug accumulation within tumor tissue due to the enhanced permeability and retention effect[19]. In a recent study, P-gp modulator-valspodar loaded methoxy-poly(ethylene oxide)-block-poly(e-caprolactone) (PEO-b-PCL) micelles were developed[20]. Intravenous administration of micelles significantly lowered the clearance and volume of distribution of valspodar and an increase in plasma area-under the curve (AUC) was observed compared to when valspodar was given in Cremophor® EL formulation. Administration of valspodar loaded micelles reduced the pharmacokinetic interactions with doxorubicin in Sprague-Dawley rats in *in vivo* studies[21].

Hence, using drug delivery system can reduce the effective doses of MDR modulator required to attenuate transporter function in drug resistant cells. A sustained release profile and an

enhanced bioavailability of these inhibitors at the pharmacological site of action can lead to higher and prolonged sensitization of drug resistant tumors. From this work we can conclude that NP formulations of dual inhibitors may as well lead to better efficacy in overcoming MDR in tissues such as the blood-brain barrier which show over-expression of multiple efflux transporters[5]. As the cell lines used in this study were of canine and human origin, it was not feasible to conduct in vivo experiments on animal models. However, it would be highly desirable to compare pharmacokinetic and MDR modulatory activity of inhibitor and their respective NP formulations in animal model for further clinical translatability.

5.6. Conclusion

Two newly identified potent BCRP/ P-gp inhibitors- KCJ-160 and KCJ-199, were formulated into PLGA-based nanoparticles. The compounds have shown higher potency against BCRP and to a lesser extent in P-gp. Nanoparticle formulations (carrying equivalent inhibitor dose) exhibited complete reversal of drug resistance in two pairs of cell lines that over-expressed each transporter (BCRP and P-gp). Both inhibitors having high potency against BCRP, showed complete sensitization of BCRP expressing cell lines when formulated as nanoparticle. However, the most promising results were against P-gp where nanoparticles performed better than the inhibitor solution of both compounds. The results clearly establish that the sensitization of cells can be achieved with sustained delivery of inhibitors as obtained from nanoparticle formulations of respective inhibitors. To the best of our knowledge this is the first report on BCRP inhibitor loaded nanomedicinal intervention. This study is also the first to evaluate dual-inhibitors loaded nanocarriers with inhibitory potential against multiple transporters (BCRP and P-gp).

5.7. References

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6. Summary and Conclusion

Failure of chemotherapy due to development of multidrug resistance (MDR) in cancer has lead to diminishing treatment options. ATP-binding cassette (ABC) superfamily of trans-membrane transporters is responsible for drug efflux of which P-glycoprotein (P-gp) is the most characterized. Following decades of research, there is little clinical benefit in terms of MDR modulation spanning three generations of MDR inhibitors. This has been attributed to pharmacological disadvantages associated with most inhibitors such as their poor specificity and low availability at the tumor site.

Nanocarriers offer promise as drug delivery vehicles of anti cancer drug/ MDR inhibitor and in overcoming drug resistance in cancer, as exemplified by many successful outcomes in last few years. Recently, certain excipients/ surfactants which are routinely applied in the pharmaceutical industry to improve solubility and bioavailability of the drug have shown ability to inhibit the MDR transporters in drug resistant tumors.

In the first part of this work, various excipient-based nanoparticles were developed. As the nanoparticle surface first to interact with cell membrane, presence of 'surface active' excipient molecule was conceptualized to modulate P-gp transporter present on the membrane as soon as the two come in contact. We observed that, Cremophor® EL and CTAB-based nanoparticles increased the sensitivity of highly resistant glioma cell lines to doxorubicin by up to 4.7-fold in comparison to the treatment with anti-cancer drug alone. SDS-based nanocarriers improved cytotoxicity marginally, Solutol® HS15 and Tween® 80 did not exhibit considerable chemosensitization as formulations.

Thus, with 'surface active' NPs, sensitization achieved was ascribable entirely to the particles devoid of any MDR inhibitors. Although, inherently active nanocarriers demonstrated potential; encapsulating MDR inhibitors can lead to synergistic and enhanced anti-MDR effects to achieve complete inhibition of ABC transporters.

In order to evaluate the effect of MDR-inhibitors, we chose inhibitors of first and third generation- Verapamil hydrochloride and Elacridar respectively; and to see the effect of surface

charge, representative of each of non-ionic, cationic and anionic excipients were used. Amongst the different categories of surfactants used, MDR inhibitor loaded CTAB nanoparticles gave very promising results with respect to reversal in drug resistant cell lines as compared to anionic and non-ionic inhibitor-loaded nanoparticles. To chalk out possible mechanism of NP uptake, various pharmacological inhibitors were used and it was observed that a caveolae-mediated endocytosis might be in effect. Nanoparticle uptake appeared to be influenced by size and not by charge, as the difference in mechanism of uptake was not significantly different amongst cationic, anionic and non-ionic particles.

The higher responses with CTAB-nanoparticles can be explained on the basis of adhesion of cationic CTAB nanoparticles to the negatively charged cell membrane which have been investigated previously and were also confirmed in this work as observed by confocal laser scanning microscopy.

Besides P-gp, breast cancer resistance protein (BCRP) are another set of efflux transporters of the ABC superfamily which were initially discovered in drug resistant breast cancer cell lines but later found to be expressed in other key tissues including blood-brain barrier and gastrointestinal tract. Research is underway to develop ABC inhibitors which can target multiple efflux transporters. However, as affinity for multiple ABC transporters seems to broaden the functionality of such dual inhibitors, the compass of potential side effects also raises. Hence, it is imperative to deliver them in nanocarriers so as to bypass the transmembrane without their recognition by drug transporters.

Recently developed quinazoline compounds KCJ-160 and KCJ-199 demonstrate high inhibitory potency against BCRP transporters and to a lesser extent P-glycoprotein. The compounds were formulated as nanoparticles, characterized and assessed in relevant BCRP and P-gp over-expressing drug-resistant & drug-sensitive cell lines. Nanoparticles offered particularly interesting results due to their ability to release encapsulated inhibitors over a prolonged period resulting in higher sensitization of drug resistant cells, in contrast to the soluble inhibitors which offered shorter duration of activity. Complete reversal of drug resistance can therefore be achieved using inhibitor-loaded nanoparticles.